Vol. 49 May 1960 No. 5

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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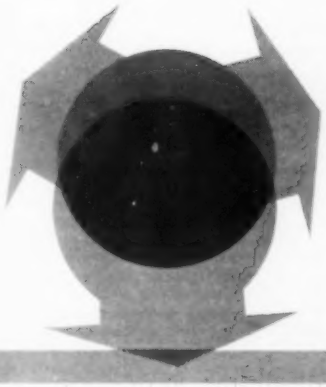

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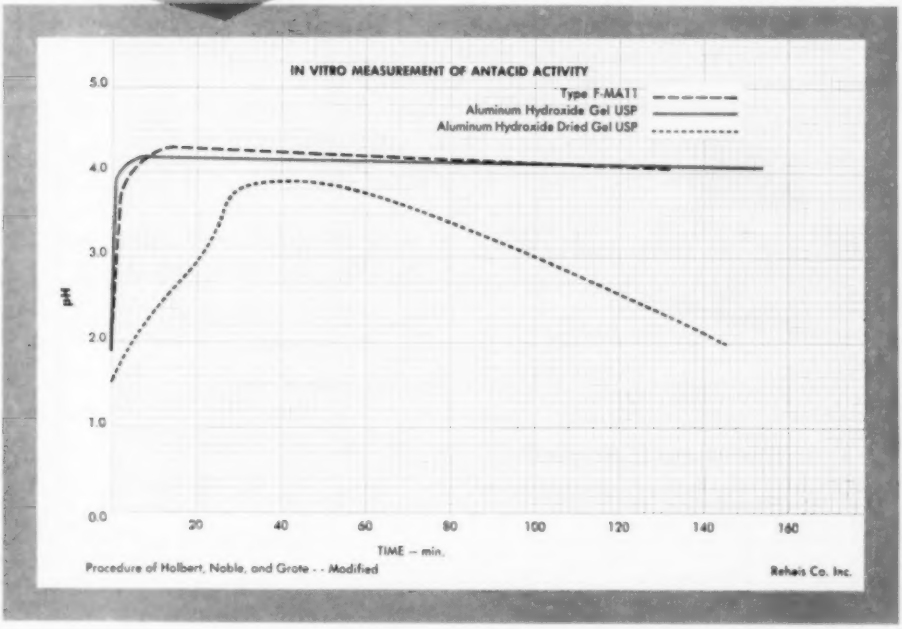

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VOLUME 49 *MAY 1960* NUMBER 5

The Synthesis and Antifungal Studies of Some Benzofuran Compounds*

By FOO PAN† and GAIL A. WIESE

An improved synthesis of benzofuran-2-carboxaldehyde and 2-methylbenzofuran-3-carboxaldehyde and the preparation of twenty-three new derivatives are reported. They were evaluated against three pathogenic fungi, together with some other derivatives which have been described in the literature. Some of these compounds showed significant *in vitro* antifungal activity. Of all the compounds tested, including the standard, undecylenic acid, benzofuran-2-carboxaldehyde exhibited high activity in every case against all three organisms. A discussion of the activity and structure relationship is based on the data resulting from this investigation. Further studies seem to be warranted.

SOME ALDEHYDES and their derivatives have been found to be effective antifungal and/or antibacterial agents. Powerful antifungal as well as antibacterial activity of decanoylacetaldehyde and related compounds was reported by Noguchi and Okeda (1). Halo-2,3-butanedione-3-oximes have been patented as antifungal agents (2) and some monosubstituted salicylaldehyde alkoximes (3) were found to be effective as fungicides in dilutions as low as one p. p. m. Ward and co-workers (4) reported the outstanding fungistatic activity of 5-nitro-2-furfural oxime and a few other nitrofuran compounds. 5-Nitro-2-furfural semicarbazone (5) has been successfully used for the treatment of various dermatological conditions and N-(5-nitro-2-furfurylidene)-1-aminohydantoin (6) has also been established as an antibacterial agent.

Benzofuran compounds remain uninvestigated as antifungal agents, probably due to the fact that so far no benzofuran compound has found application in pharmaceutical chemistry (7). It is interesting to note, however, that griseofulvin, an orally effective (8) antifungal antibiotic, is a carbonyl derivative of 2,3-dihydrobenzofuran (9) and that some derivatives of thianaphthene (10), a sulfur analog of benzofuran, have been found to possess considerable antifungal activity.

In view of the facts outlined above, two benzofurancarboxaldehydes and a number of their derivatives have been synthesized and screened *in vitro* for their antifungal activity against *Microsporum audouini*, *Microsporum gypseum*, and *Trichophyton rubrum*, by agar-plate, paper-disk technique. Some of the more active and the less soluble compounds were tested in the form of ointment. Undecylenic acid was used in both instances as a standard.

The compounds prepared and tested are shown in Table I. The results of the antifungal studies are listed in Tables II and III.

All 28 derivatives, including 23 new compounds, were prepared by condensation reactions involving the aldehyde group. The condensing agents included various aromatic and heterocyclic amines, isoniazid, aminoguanidine, and acetone, as well as the usual characteristic

* Received August 13, 1959, from the State University of Iowa, College of Pharmacy, Iowa City.
Abstracted from a thesis submitted by Foo Pan to the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of Master of Science, August 1959.
† Fellow of the China Medical Board of New York, Inc., 1958–1959. Present address: Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan, China.

TABLE I.—BENZOFURAN COMPOUNDS SYNTHESIZED AND TESTED

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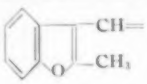
R′ = 

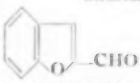
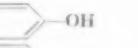
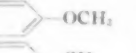
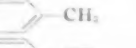
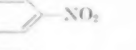
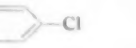
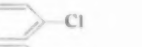
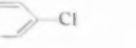
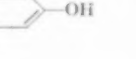
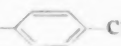
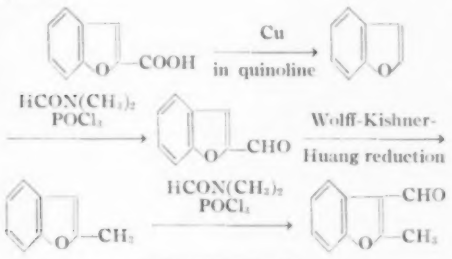
No.	Structure	Appearance	M. p., [b] °C.[a]	Yield, %	Nitrogen, [b] % Calcd.	Found	Reference[c]
I		...	...	..	...	...	12, E
II	R=O·NaHSO$_3$	...	...	..	...	...	13, E
III	R=NNH-	...	...	..	...	...	14
IV	R=NNHCONH$_2$	...	...	..	...	...	14
V	R=NNHCSNH$_2$	...	...	..	...	...	13
VI[d]	R=NNH-	Brownish red needles	213–214	73	14.94	14.88	E
VII[d]	R=NOH	White needles	131–132	80	8.69	8.64	E,
VIII[d]	R=NNHC-	Colorless prisms	200–202	71	15.85	15.71	E
IX[d]	R=NNHC(NH$_2$)(NH·HCl)	White crystals	176–178	15	23.48	23.73	E
X[d]	R=N-	Pale yellow crystals powdered	56–56.5[e]	46	6.33	6.17	E
XI[d]	R=N- 	Bright yellow needles	230–231 (Decompn.)	42	5.91	5.79	E
XII[d]	R=N- 	Yellow crystals powdered	89–90	65	5.58	5.49	E
XIII[d]	R=N- 	Bright yellow fine crystals	95–96.5	62	5.96	5.82	E
XIV[d]	R=N- 	Bright yellow crystals powdered	176–177	55	10.53	10.27	E
XV[d]	R=N- 	Pale yellow needles	138–139.5	71	5.48	5.45	E
XVI[d]	R=N-	Pale yellow crystals powdered	109–111	23	12.61	13.12	E
XVII[d]	R=N-	Yellow crystals powdered	131–132	70	10.29	9.94	E
XVIII[d]		Pale yellow crystals powdered	138–139	36	10.08	10.24	E
XIX[d]	R=CHCOCH$_3$	Yellow needles	97–98[f]	22	C: 77.42 H: 5.38	C: 77.35 H: 5.17	E
XX[d]	R=N-	Shining yellow scales	133.5–134.5	70	5.02	5.00	E
XXI[d]	R=N- 	Yellow crystals powdered	92–93	60	4.83	4.70	E
XII[d]	R=N- 	Redish yellow needles	112–112.5	40	9.32	9.18	E
XXIII		...	...	..	...	...	11, E
XXIV[d]	R′=N- 	Greenish yellow prisms	130–131	21	5.58	5.34	E

TABLE I (*Continued*)

No.	Structure	Appearance	M. p., ° C.[a]	Yield, %	Nitrogen,[b] % Calcd.	Nitrogen,[b] % Found	Reference[c]
XXV[d]	R′=N–  –Cl	Bright yellow scales	77.5–78.5	46	5.19	5.04	E
XXVI[d]	R′=NNHCSNH$_2$	Yellow crystals powdered	231–232 (Decompn.)	56	18.01	17.43	E
XXVII[d]	R′=NNHC(=O)–	Pale yellow needles	203–204	58	15.05	14.83	E
XXVIII[d]	R′=NOH	Pale yellow needles	114–115	74	8.00	7.87	E
XXIX[d]	R′=NNH–	Bright yellow platelets	129–130	89	11.20	11.08	E
XXX	R′=NNHCONH$_2$	...	...	...	...	...	11

[a] Determined by capillary method and uncorrected. [b] Carbon and hydrogen for Compound XIX. [c] E, experimental. [d] New compound. [e] Boiling point, 164°/1 mm. [f] Boiling point, 128°/1 mm.

reagents for carbonyl compounds. The process used for preparing the two benzofurancarboxaldehydes is indicated below. The procedure of Bisagni and co-workers (11) for the formylation of benzofuran was modified to give improved yields.



EXPERIMENTAL

Synthesis[1]

Benzofuran-2-carboxaldehyde(I).—To a mixture of 37.5 Gm. of benzofuran, b.p. 59–61° (6 mm.), prepared from coumarilic acid in 81% yield by a modified procedure of Tanaka (15), and 27.6 Gm. of *N,N*-dimethylformamide, 55.2 Gm. of phosphorus oxychloride was added in small portions with shaking, while the temperature was kept below 50°. The reaction mixture was allowed to stand at room temperature overnight. It was heated gradually to about 95° and then kept at 93 ± 2° for nine hours, with occasional shaking. It was again allowed to stand overnight. The mixture was poured into 500 Gm. of ice and water with stirring, and extracted with three 200-cc. portions of ether. The ethereal extract was washed twice with 150-cc. portions of 5% hydrochloric acid. These aqueous washings were in turn extracted with 100 cc. of ether. The combined ethereal extracts were washed with a saturated sodium bicarbonate solution and then with water. When the ethereal solution was shaken in a mechanical shaker with a solution of 180 Gm. of sodium bisulfite in 300 cc. of water, a white addition product of benzofuran-2-carboxaldehyde deposited. The precipitate was collected on a filter and washed successively with alcohol and ether.

The aldehyde also may be obtained from the ethereal solution, after drying over anhydrous sodium sulfate and removing the solvent, by fractional distillation under reduced pressure. The yield of the free aldehyde, b. p. 90–93° (1 mm.), n_D^{25} 1.6334, or its sodium bisulfite adduct, was 67–71%.

Benzofuran-2-carboxaldehyde *p*-Nitrophenylhydrazone (VI) and Benzofuran-2-carboxaldoxime-(VII).—Both of these compounds were prepared from 0.01 mole of the aldehyde by the usual method (16) and recrystallized from diluted alcohol.

Benzofuran-2-carboxaldehyde Isonicotinoylhydrazone(VIII).—A solution of 2.92 Gm. (0.02 mole) of the aldehyde in 12 cc. of alcohol was mixed with a solution of 2.75 Gm. of isoniazid in 12 cc. of alcohol and boiled for three minutes. The crystals which deposited after cooling were filtered and recrystallized from alcohol.

Benzofurfurylideneaminoguanidine Hydrochloride(IX).—A solution of 0.02 mole of benzofuran-2-carboxaldehyde in 50 cc. of alcohol was mixed with 2.8 Gm. of aminoguanidine bicarbonate and 5.5 Gm. of sodium acetate. The mixture was heated on a water bath at about 60° for one hour, acidified to Congo red with concentrated hydrochloric acid, and filtered. The filtrate was concentrated to about one-half its original volume at room temperature and then cooled to about −10°. The precipitate which formed was collected and recrystallized three times from absolute alcohol.

Benzofurfurylideneaniline(X).—The aldehyde, 0.02 mole, was mixed with 2 cc. of freshly redistilled aniline in a small evaporating dish. The mixture was heated on a boiling water bath for twenty minutes, cooled with stirring, and washed thoroughly with *n*-pentane. The solid was filtered and recrystallized from ether-*n*-pentane mixture.

Benzofurfurylidene-*p*-aminophenol(XI).—To a solution of 2.18 Gm. of *p*-aminophenol in 10 cc. of absolute alcohol, 0.02 mole of benzofuran-2-carboxaldehyde was added with stirring. Reaction took place almost instantaneously. The mixture was heated under reflux for thirty minutes, cooled, and filtered. The product was recrystallized from methanol.

[1] Properties and yields of the new compounds prepared are shown in Table I.

TABLE II.—ANTIFUNGAL ACTIVITY OF SOLUTIONS OF BENZOFURAN COMPOUNDS[a]

Compound No.[b]	*M. audouini* 1%	*M. audouini* 5%	*M. gypseum* 1%	*M. gypseum* 5%	*T. rubrum* 1%	*T. rubrum* 5%
Solution in Water						
II	0	1	0	9	3	12
Water	0	0	0	0	0	0
Solutions in Alcohol						
I	12	[c]	7	[c]	13	[c]
V	0	..[d]	0	..	1	..
VIII	0	..	0	..	0	..
IX	5	11	3.5	12	2	15
X	0	4	2.5	20	1	10
XII[e]	0	0	0	0.5	0	2
XIII	0	0	0	6	0	1.5
XXIV	0.5	23.5	0	[c]	0.5	20
XXVIII	7	9	6	10	6	8.5
Undecylenic Acid	6	8	2	9.5	6.5	13
Alcohol	0	0	0	0	0	0
Solutions in Acetone						
III	0	0	1	1	1	2.5
VI	0	0	0	0	0	0
VII[e]	2	12	3	12	3	9
XI	0	..	0	..	0	..
XV[e]	0	0	0	0	0	0
XVI[e]	0.5	12	1	17.5	1	15.5
XVII	0	0	0	0	0	2
XVIII	0	..	0	..	0	..
XIX	2	5	1.5	3.5	3	6
XX	0	..	0	..	0	..
XXI	0	1	0	2	0.5	0.5
XXII	8.5	12	6.5	10	2	10
XXIII	0	10	1	19	0.5	7.5
XXV	0	3	0	4	1	1.5
XXVI	0	..	0	..	0	..
XXVII	0	..	0	..	0	..
XXIX	3.5	8	1	7	3	12
Acetone	0	0	0	0	0	0

[a] Zones of inhibition in mm.
[b] The formulas of the compounds are listed in Table I, and their names in the experimental section.
[c] Little or no growth after 15-day incubation.
[d] Not tested.
[e] 4% solution instead of 5%.

Benzofurfurylidene-*p*-anisidine(XII) and Benzofurfurylidene-*p*-toluidine(XIII).—These two compounds were prepared similarly to compound XI except that the heating time was two hours and the products were recrystallized from isopropyl alcohol.

Benzofurfurylidene - *p* - nitroaniline(XIV).—This compound was prepared in the same manner as compound XI, except the reactants were heated under reflux in 20 cc. of absolute alcohol for four hours and the product recrystallized from benzene.

Benzofurfurylidene-*p*-chloroaniline(XV).—To a solution of 0.02 mole of the aldehyde in 40 cc. of ether, 2.55 Gm. of *p*-chloroaniline was dissolved with stirring. Crystallization took place within a few minutes. After allowing to stand at room temperature for three hours, the mixture was filtered and the product recrystallized from alcohol.

Benzofurfurylidene-2-aminopyridine(XVI).—Equimolar amounts (0.02 mole) of the aldehyde and 2-aminopyridine were heated under reflux in 5 cc. of absolute alcohol for four hours. The crystals formed after cooling were filtered, washed with ether, and recrystallized several times from diluted alcohol.

TABLE III.—ZONES OF INHIBITION IN MILLIMETERS OF 5% OINTMENTS OF BENZOFURAN COMPOUNDS[a]

Compound No.[b]	*M. audouini*	*M. gypseum*	*T. rubrum*
I	[c]	[c]	[c]
II	0	1	3
IV	0	0	0
V	0	0	2
VII	5	8	9
VIII	0	0	0
IX	5	5	8
X	3	4	8
XI	0	0	1
XIV	0	1	4
XVI	1	12	8
XVIII	2	1	5
XX	0	1	2
XXII	5	8	11
XXIII	5	12	6
XXIV	2	7	4
XXVI	0	0	0
XXVII	0	0	0
XXVIII	4	7	8
XXX	0	0	1
Undecylenic acid	4	3	8
Hydrophilic ointment, U. S. P.	0	0	0

[a] Only the more active and the less soluble compounds were tested in ointment form.
[b] The formulas of the compounds are listed in Table I, and their names in the experimental section.
[c] Little or no growth after 15-day incubation.

Benzofurfurylidene - 3 - aminoquinoline(XVII).—This compound was prepared in the same manner as compound XI, except the reactants were heated under reflux in 10 cc. of absolute alcohol for four hours and the product recrystallized from diluted alcohol.

Benzofurfurylidene-2-aminobenzothiazole-(XVIII).—This compound was prepared in the same manner as compound XI, except the product was recrystallized from alcohol.

Benzofurfurylideneacetone(XIX).—To a mixture of 20 cc. of C. P. acetone and 0.04 mole of benzofuran-2-carboxaldehyde in a 250-cc., glass-stoppered reagent bottle, 2 cc. of 10% sodium hydroxide solution was added dropwise while the temperature was kept at 25–35°. The bottle was stoppered and shaken in a mechanical shaker at room temperature for three hours. The reaction mixture was acidified to litmus with diluted hydrochloric acid and the two layers were separated in a separatory funnel. The upper aqueous layer was extracted with 10 cc. of benzene and the benzene extract combined with the organic layer, washed with water, and dried over anhydrous sodium sulfate. After removal of the solvent, the product was purified by sublimation under reduced pressure.

Methyl Benzofurfurylidene-*p*-aminobenzoate-(XX).—This compound was prepared in the same manner as compound XI, except the reactants were heated under reflux in 5 cc. of absolute alcohol for one hour. The crude product was washed with ether and recrystallized from methanol.

Benzofurfurylidene - 2,4 - dichloroaniline(XXI).—A mixture of 0.02 mole of the aldehyde and 3.24 Gm. of 2,4-dichloroaniline was heated under reflux in 5 cc. of absolute alcohol for two hours and then

cooled by ice and water. The crude product, deposited as a heavy oil layer, was separated from most of the solvent by decantation and dissolved in ether. Crystallization took place when the ethereal solution was stirred and cooled by a dry ice and acetone mixture. The crystals formed were collected on a filter and recrystallized from diluted alcohol.

Benzofurfurylidene-2-nitro-4-chloroaniline(XXII).—This compound was prepared in the same manner as compound XI, except the reactants were heated under reflux in 5 cc. of absolute alcohol for two hours and the product recrystallized from dilute alcohol.

2-Methylbenzofuran.—This compound, b. p. 192–194°, was prepared in 85% yield by the procedure of Bisagni and co-workers (11), using 0.1 mole of benzofuran-2-carboxaldehyde, 50 cc. of diethylene glycol, 15 Gm. of 99–100% hydrazine hydrate, and 15 Gm. of potassium hydroxide.

2-Methylbenzofuran-3-carboxaldehyde(XXIII).—2-Methylbenzofuran (17.7 Gm.) was formylated with N,N-dimethylformamide (10.6 Gm.) and phosphorus oxychloride (23 Gm.) by the procedure described for the preparation of benzofuran-2-carboxaldehyde except the reaction mixture was heated at 93 ± 2° for eleven hours and the product isolated as the free aldehyde by concentrating the dried ethereal extract and recrystallizing the crystals from petroleum ether. The yield of the yellow needles, m. p. 80.0–80.5°, was 16 Gm. (75%).

2-Methyl-3-benzofurfurylidene-*p*-aminophenol-(XXIV).—Three grams of 2-methylbenzofuran-3-carboxaldehyde and 2.1 Gm. of *p*-aminophenol were dissolved in 10 cc. of hot absolute alcohol and heated under reflux for one and one-half hours. The product, failing to crystallize after the reaction mixture was allowed to stand in a refrigerator overnight, was cooled with a dry ice and acetone mixture. The product which precipitated was recrystallized first from methanol and then from benzene.

2-Methyl-3-benzofurfurylidene-*p*-chloroaniline-(XXV).—A solution of 2 Gm. of 2-methylbenzofuran-3-carboxaldehyde and 1.65 Gm. of *p*-chloroaniline in 14 cc. of absolute alcohol was heated under reflux for two hours and then allowed to stand in a refrigerator overnight. The filtered precipitate was recrystallized from alcohol.

2-Methylbenzofuran-3-carboxaldehyde Isonicotinoylhydrazone(XXVII).—This compound was prepared as the benzofuran-2-carboxaldehyde analog from 1.92 Gm. (0.012 mole) of 2-methylbenzofuran-3-carboxaldehyde and an equimolar amount of isoniazid.

2-Methylbenzofuran-3-carboxaldehyde Thiosemicarbazone (XXVI), 2-Methylbenzofuran-3-carboxaldoxime(XXVIII), and 2-Methylbenzofuran-3-carboxaldehyde Phenylhydrazone(XXIX).—These compounds were prepared by the usual procedure (16), using 0.015 mole of the aldehyde. Compounds XXVI and XXVIII were recrystallized from diluted alcohol, while compound XXIX was recrystallized from alcohol.

Antifungal Section

The compounds prepared were screened *in vitro* for antifungal activity against *Microsporum audouini*, *Microsporum gypseum*, and *Trichophyton rubrum* in solutions and/or ointments, using undecylenic acid as a standard.

An agar-plate, paper-disk technique, similar to that reported by Goettsch and Wiese (10), was used for the testing of the compounds in solutions. Sabouraud's dextrose agar, pH 5.6, with the addition of 0.1% of Tween 80, was used as the culture medium. The compounds were tested in 5% and/or 1% solutions in acetone or alcohol, according to the individual solubilities. In a few cases, 4% solutions were used instead of 5%. Water was used as solvent in one case. Acetone and alcohol were tested as controls. Two disks, 12.7 mm. in diameter, were placed on each culture plate and duplicate plates were run in each case. The zones of inhibition were measured after the plates were incubated at room temperature (25–29°) for seven days. Averages of four readings are listed in Table II.

The same method was used for the preparation of culture plates when ointments instead of solutions were used for testing. Five per cent ointments of those compounds, which were less soluble in the solvents used or showed higher activity in solutions, in hydrophilic ointment U. S. P., with no preservative, were prepared and placed in tubes under aseptic conditions. Two streaks of the ointment, about 50 mm. in length and 100 mg. in weight, were placed separately on each plate. Two plates were used for each compound. The ointment base was included in testing as control. These plates were also incubated at room temperature for seven days. Averages of the zones of inhibition, measured as the minimum distance between the periphery of the ointment and the colony growth, are in shown Table III.

DISCUSSION

Benzofuran-2-carboxaldehyde has been prepared from 2-methylbenzofuran by oxidation with selenium oxide (12), from the glyoxal compound derived from coumarilic acid (14), and from coumarilic acid anilide (13). The last method has been tried and proved satisfactory only on a very small scale. For the preparation of this compound, the authors have modified the procedure of Bisagni and co-workers (11). By modifying both the reaction conditions and the process of separation and purification, the yield has been improved, with 71% of the theoretical yield obtained as compared to a reported 37% yield. The procedure for preparing 2-methylbenzofuran-3-carboxaldehyde (11) has been similarly modified.

2-Methylbenzofuran, the intermediate for 2-methylbenzofuran-3-carboxaldehyde, was prepared in 85% yield by the Wolff-Kishner-Huang reduction of benzofuran-2-carboxaldehyde according to the procedure of Bisagni and co-workers (11). The poor yield originally reported is doubtful.

The procedure for the decarboxylation of coumarilic acid reported by Tanaka (15) has been modified in two aspects with favorable results. First, only one-third the quantity of quinoline was used, and secondly, the product was isolated and purified directly by fractional distillation.

As to the synthesis of the new benzofuran compounds, no difficulty was encounted in the preparation of common derivatives of aldehydes such as oximes, hydrazones, and thiosemicarbazones by the usual methods.

There were considerable amounts of resinous by-

products formed, probably from the alkali-catalyzed polymerization, when benzofurfurylideneacetone (XIX) was prepared. No suitable solvent for satisfactory crystallization of this compound was found; so sublimation under reduced pressure was finally employed to purify the product. Perhaps this side-reaction could be inhibited to some extent by conducting the reaction at lower temperatures.

The difficulties encountered in purifying the aminoguanidine and aminopyridine derivatives(IX, XVI) account for the poor yields.

In the preparation of the aniline derivative(X), it was found imperative to obtain crystals from the reaction mixture by cooling, washing, and filtering before any further purification could be accomplished. Otherwise, a pure product could not be obtained even by vacuum distillation.

The results of the *in vitro* antifungal studies, as shown in Tables II and III, indicate that most of the compounds tested exhibited some activity against one or more strains of fungi. Generally, nine compounds (I, VII, IX, X, XVI, XXII, XXIII, XXIV, and XXVIII) showed equal or greater activity than undecylenic acid, which is a frequently used standard antifungal agent. Solubility of the individual compound seems to be one of the factors which influence the activity, since none of the least soluble compounds exhibited significant activity even in ointments. In this connection it is interesting to note that compound XI was practically inactive while its analog, compound XXIV, which was much more soluble, was among the most active compounds.

Of all the compounds tested, benzofuran-2-carboxaldehyde(I) was most active in every case. 2-Methylbenzofuran-3-carboxaldehyde(XXIII), although more active than undecylenic acid against two of the three organisms used, was less active than benzofuran-2-carboxaldehyde. Chemically they are different in two ways, i. e., the position of the aldehyde group and the presence of a methyl group in XXIII. It is thought that the methyl group greatly decreases the polarity of the carbonyl group, and probably this factor plays a more important role in decreasing its antifungal activity. Although no conclusion should be drawn without further studies in this field, the relationship between the activity and polarity of antifungal compounds have been noted in several other studies (17–19).

Until more of the related compounds are prepared and studied, it is difficult to say whether the benzofuran nucleus or the aldehyde group contributes more to antifungal activity, or if both are essential. Although the antifungal antibiotic, griseofulvin (8) is a carbonyl derivative of benzofuran, Ward and co-workers (4) noted no structural specificity in the nitrofuran compounds studied.

The activity shown by a number of other compounds tested is not necessarily due to reformation of the parent aldehyde, since among seven derivatives of compound XXIII studied, three compounds (XXIV, XXVIII, and XXIX) exhibited somewhat greater activity than the parent compound in one or more cases.

Among the usual derivatives of the two aldehydes, both oximes are quite effective. This agrees with the fact that most of the functional derivatives of aldehydes reported to possess antifungal properties were oximes (2–4). While one phenylhydrazone (XXIX) showed moderate activity against all three organisms, another(III) was only very weakly active against two organisms. All the semicarbazones (IV, XXX), thiosemicarbazones(V, XXVI), and *p*-nitrophenylhydrazone(VI) tested showed little or no activity.

Both of the isonicotinoylhydrazones(VIII, XXVII) were inactive against the fungi. The unsaturated ketone(XIX) formed by the Claisen reaction was somewhat active. The aminoguanidine derivative(IX) was one of the most active compounds in this series. It may be interesting to point out that another antifungal antibiotic, Eulicin, is a guanidino compound (20).

Of the imines, or Shiff's bases, prepared from the various aromatic and heterocyclic amines, the aniline and aminopyridine derivatives(X, XVI) were among the most active agents found in this investigation. Except in the case of compound XXIV, no monosubstituted aniline derivative showed significant activity. Compound XXII, however, with both chlorine and nitro groups on the benzene ring, indicated an enhanced activity.

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Mixing of Pharmaceutical Solids: The General Approach*

By DAVID TRAIN

IT IS IMPORTANT to have a suitable criterion for good mixing for pharmaceutical solids, because the attainment of an accurate dosage of a small quantity of a potent drug so often depends on the thoroughness by which it is dispersed through the bulk of another, or other materials. Mixing of solids, of necessity, presupposes the particulate form, and there are certain factors and properties associated with particles that will always affect the results of a mixing operation. It is the purpose of this paper to review these factors and properties, and also the operation, so that desirable criteria and possible practical procedures may be assessed rationally when a specific problem in solid mixing is being considered.

Consider an assemblage of particles or a static powder bed before any mixing process begins. All particles are subject to a constant force of gravity and they are in some sort of spatial equilibrium with one another. As long ago as 1885 Osborne Reynolds (1) showed that in order to obtain relative particulate movement within such a bed, the volume of the bed must be increased. Subsequent work by Jenkin (2), and by Brown and Hawksley (3) have amply confirmed that even in the case of a randomly packed bed, no appreciable movement can take place while the bed is in the condition that it spontaneously takes up as it is formed. Brown and Hawksley found that movement in a bed was produced by failure along a slip line or, in other words, a shear movement. For such a movement to take place, there must be sufficient space between the particles and this provides the first necessary condition for mixing. Assuming there is a preliminary condition of partial or total segregation, it follows that mixing must be achieved by some form of interparticulate movement, and this can only take place if the bed of particles is expanded. The extent to which a powder mass may be made to dilate is a complex function of the physical properties of the materials forming the system, but a good empirical test is to compare the specific volume of a tapped bed (4) with the specific volume of the same bed which is on the point of being fluidized. The ease with which movement could take place is, within reason, a direct function of the degree of expansion which can be produced in the specific volume of the particulate system. Practically, this means that there should be sufficient room in a mixing container to allow the powder mass to dilate, and it also points to the risk of loss of economic efficiency if the space in a given mixer is overfilled.

Assuming interparticulate movement is possible, then, in order to produce such movement, suitable forces must be applied to the particles. Since continued application of pure tensile or compressive forces will serve only to increase or decrease the specific volume of the system without the particles changing stations relative to one another, it follows that shear forces will be necessary to produce interparticulate movement. See Fig. 1. Because the force of gravity is constantly applied, a vertical force either reduces or reinforces the vertical stress according to whether its direction is upwards or downwards. Any horizontal component of force acts at right angles to the gravitational force, and will, in combination with the vertical force, automatically induce a shear stress into that part of the system. If the system is incapable of resisting such a stress, movement along a slip plane takes place.

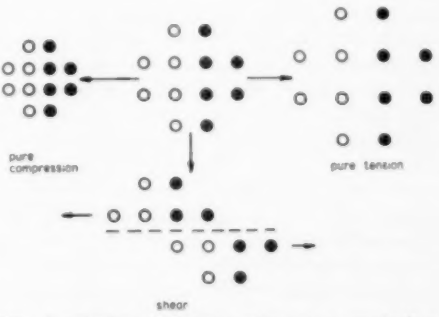

Fig. 1.—Action of forces acting on a particulate system.

The forces are introduced by some external means and, by interparticulate transmission, act within the bed. To ensure mixing there must be such movement that each and every particle could visit, if given time, every point within the con-

* Received August 21, 1959, from the School of Pharmacy, University of London, England.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August, 1959.

lines of the system in its expanded state. This requires a three-dimensional stress system which will induce movements in all planes. To ensure rapid, and therefore economic, mixing it is necessary to cause the greatest possible number of slips per unit of time. If adjacent slip planes are produced so that they are not more than one particle apart then there will be movement between all particles.

These considerations give, *a priori*, requirements of any apparatus to be used for mixing solid particles. The specific volume of the system must be suitably increased to give freedom of movement to the particles. Forces must act so that a three-dimensional grid would be necessary to plot the lines of movement of each and every particle. These forces must be such that, in time, each particle could visit every point within the confines of the particulate system in its expanded condition. Finally, when all movement ceases the system must be able to take up a state of static equilibrium without segregation of particles taking place. Applied to any specific situation three possible causes of inefficient mixing are highlighted: (*a*) Insufficient dilation of the bed, either generally or locally; this causes hindrance to particle movement and in its mildest form causes increased time for mixing, and under the worst conditions would prevent any possibility of proper mixing being achieved. (*b*) Induced forces being insufficient to produce suitable movement in all directions, thus leaving portions of the assemblage undisturbed. This can be manifest as movement in two axes only as in a simple cylindrical drum revolving horizontally about its axis when there is little or no lateral particulate movement. Inadequate forces may also allow the presence of dead zones within a system, or may allow aggregates (loose assemblages of particles held together by light cohesive surface forces such as those caused by water films or electrostatic charges) to move round as compound entities, within which no relative movement or particle exchange takes place. (*c*) Preferential movement of a certain type of particle due to dissimilarity of physical property (e. g., size or density) of materials being mixed. This can be the cause of imperfect mixing, however long the operation is allowed to proceed, or segregation as soon as mixing conditions change, such as stopping at the end of the process.

EFFECTS PRODUCED BY DISSIMILAR PARTICLES AND SURFACE-ACTIVE FORCES

Before considering the criteria for mixing, it is necessary to summarize the effects produced by certain physical conditions which can occur in the materials to be mixed.

First, there is the difference in size between particles. Coulson and Maitra (5) have reported careful investigations using a simple drum mixer with its axis set at an inclination of 23° to the horizontal because this angle gave best mixing conditions. They found that if fine particles were put at the bottom of the drum and coarse particles of the material placed on top, no mixing occurred. When the position of the sizes was reversed, the larger being on the bottom, then mixing did occur for a short time but segregation developed as the coarse particles rose to the top of the bed. The reason for this difficulty of producing or maintaining a mix with particles of different sizes is due to the small particles being able to fall through the void spaces between the large ones. See Fig. 2. A small particle, $d = 0.41D$, can just slip between four larger particles touching in a square grid, while a particle $d = 0.15D$ can just slip between three larger particles touching on a triangular grid.

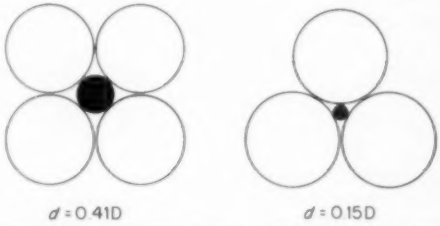

Fig. 2.—Small particles slipping between large particles.

Practically, it must be recognized that with any mixing apparatus, there is always a tendency for segregation if there are large differences in particle sizes. It is also important to note that even if a true mixture is obtained, the condition is unstable and will separate rapidly if subjected to vibration in storage or subsequent processing. Thus, although it must be accepted that some mixtures have to be made with materials of different sizes, it should always be the aim to mix components with the same size range and the closer the range, the simpler the operation.

A second condition may be caused by a density difference between the component materials. The effect is not so marked as that of difference in size, but Coulson and Maitra showed that the rate of mixing was impeded by an increase in density difference. This was supported by Gray (7) who also found that there was a tendency for segregation to develop with time of mixing in the case of certain systems, like the concrete mixer.

The prevention of segregation in this case requires a condition of vigorous movement between the particles coupled with a restriction on freedom to move in order to prevent segregation under free falling conditions. This is a difficult specification to comply with in practice. Machines which reverse the gravitational field help to reduce the adverse effect of density difference. Again vibration during subsequent processing or storage will help to produce segregation of the mixture.

The effect of particle shape on mixing conditions has not been extensively investigated, but it is well known that roughly equidimensional particles are easier to mix than any other shape. The addition of flat or acicular particles prolongs the mixing time because of their tendency to bundle, and vigorous treatment is often required to break up such assemblages.

The fourth condition is the part played by surface-active forces so that groups of particles are held together as aggregates and consequently do not disperse evenly through the other components. The forces which produce this are mainly surface tension due to adsorbed liquid films, electrostatic charges, or possibly to weak Van der Waals forces. Since the forces act on the surface of the particle, their effect becomes greater as the specific surface area of the solid increases, that is, as the absolute particle size becomes smaller, and produces powders which have high angles of repose and poor flowing characteristics. Andreasen (8) made some investigations into the "stickability" of powders and found that, in those materials which had a propensity to aggregate, the effect became very marked with particles smaller than 10 μ. This figure is quite arbitrary but it is a useful one to use because it conveniently provides an upper size limit of what could be subjectively called a "very fine powder." Neumann (4) was interested in setting up an index of "stickiness" and she achieved this by a technique developed by Hawksley (9). Briefly, she found out how much of a course granular material she required to add to a given sticky powder in order to make the resultant mixture have certain flow characteristics so that it would maintain a steady flow through a standard orifice. The results for a selection of powders is given in Table I.

TABLE I.—STICKINESS OF POWDERS

Material	Mean size, μ	Sand Required to Produce Free Flow, %
Catalyst microspheres	25	0
Calcium fluoride	30	0
Anhydrous sodium carbonate	100	23
Titanium dioxide	0.5	26
Refined kaolin	2	1,200
Natural fuller's earth	0.1	25
Activated fuller's earth	5	60
Cement	10	100
Flour	25	30

To explain the change in conditions produced in the powder, Andreasen postulated that the fine particles were adsorbed onto the surface of the large particles and as the mass of a large particle was sufficient to overcome aggregation by surface forces, the material became free flowing. As an investigation, Andreasen's experiments were successful, but the inferences must be applied with care to the mixing of pharmaceutical powders. For example, there is no evidence that all the aggregates of fine powder are broken up when sufficient coarse material has been added to make the mixture flow. Thus, compound particles can still exist and dispersal on a microscale is patchy and irregular even though the random distribution of the large particles through the mix may be perfect. Also, Andreasen was concerned with making a powder with poor flow qualities free flowing; he did very little to investigate the extent of segregation which can be produced with such a mixture under suitable conditions of subsequent processing and storage.

With the foregoing as a basis it is now possible to select a few examples to illustrate some of the inherent limitations in performance of various types of mixing equipment. Reference has already been made to the poor mixing ability of a simple cylindrical drum revolving about its axis because most of the individual particulate movement within is in one plane only. Various mechanical devices have been introduced from time to time to overcome this weakness. The addition of helical flights in one form or another was probably the first modification, and of recent years, revolving the drum with its axis at an angle to the horizontal, or on an axis which is at an angle to an axis of symmetry and to the direction of gravity, has been used successfully on a commercial basis. Into this category, too, may be placed the rotating cube-type mixer. The Z-blade mixer and planet mixer exhibit other variations in that movement is induced by impellers, while the container remains stationary. All techniques induce a skew movement on the particles as they fall under the influence of gravity. This enhances the mixing quality of the system, and is usually successful for mixing free flowing particles of even density. If density differences have to be accommodated then mixers producing a periodic abrupt reversal of flow of the powder, such as the double, V-, or Y-cone

mixers with baffles are the equipment of choice. However, in all the types of plant mentioned above it must be remembered that intensity of shear between particles is mild, and while little or no attrition of other particles may be a desirable property in the case of blending free flowing powders, the break-up of closely held aggregates of small primary particles of a specific ingredient is an inefficient process.

Because size reduction equipment relies almost exclusively on the principle of shear to break down solid material, it is to be expected that most types of plant used for this process have, at one time or another, been utilized for mixing powdered solids. Shear forces in mixing are especially important to help the break-up of the compound aggregates into primary particles. The use of such plant, however, means reducing in size by mechanical breakage at least a proportion of the particles. That this will happen is often anticipated and in such cases ingredients, which are initially oversize, are fed in the correct proportions into the plant and are reduced and partly mixed in one operation. It must be emphasized that although size reduction machinery is used for the mixing process because of the shear forces used, this does not necessarily make such plant good mixing apparatus; there may be efficient incidental mixing on a local scale, but general mixing either does not take place or if it does, proceeds slowly and may therefore be uneconomic.

Muller-type reducers, as exemplified by the pestle and mortar, the end or edge runner mills, or the buhr-stone mill, produce a shearing action between two surfaces moving parallel to the plane of contact. Only a small portion of the material is being processed at any one time and general mixing is slow because the bed is virtually in an unexpanded state and there is little relative movement in the bulk of the particles except near the zone of the muller.

With size reducing plant using impact principles, such as the pin disk mill, the hammer mill, or the fluid energy mill, intense shear forces on a local scale produce a breakdown to primary particles and turbulent air currents ensure mixing of any particles which happen to be in the plant at the time. However, the hold-up capacity of such machines is usually low, so that little or no general mixing is possible and therefore this must be done by other means.

Sieving and sifting techniques have often been recommended as suitable for aiding a mixing process, on the basis that a seive helps to break up an aggregate or a concentration of an ingredient into primary particles, or, alternatively, the meshes act as simple proportioning device and redistribute the material as it lands on the sieve. This second premise is probably correct and is a useful procedure when the size of the holes is large compared with the dimensions of the particles. The basis for breaking down aggregates is not so well founded however, because a mesh size must be selected which is only a little larger than the large particles. Since, on a probability basis, the smaller particles will tend to pass through first, it follows that there will always be a segregation on a particle size basis during the process, thus defeating the object of mixing.

PHARMACEUTICAL REQUIREMENTS OF THE MIXING OPERATION

Having set out the principles of mixing and briefly reviewed the limitations of the apparatus used to achieve it, it is necessary to consider the use to which this operation is put. Pharmaceutical materials are being mixed and therefore, by implications, are essentially for medicinal or veterinary use. The physician or veterinarian prefers to use medicaments which produce precise and dependable effects. This can only be achieved by using carefully standardized materials in exact quantities, and the pharmaceutical world has built up a reputation for supplying most of its dosage forms in this manner by suitable process and analytical control. To be successful such control has to be used intelligently, and this applies especially in the case of any dosage forms involving the use of mixtures of powders. Much has been said and written about the procedure for sampling particulate matter when every effort is made to obtain a representative sample of a batch of material. Such routines are correct when a commercial transaction is involved, for it is an assessment of the average content of the ingredients that is required. But when a powder is to be used as the basis for a medicine, then it is the actual content of a specific single dose which must be the criterion for control and sets what has aptly been named "the scale of scrutiny" (6).

The concept may be clearer to understand if one quotes a few examples: the scale of scrutiny for a foot powder or a dusting powder is the amount of an average application; for an internal medicine it is the dose unit or minimum normal dose, a teaspoonful of effervescent granules, the contents of a capsule or cachet, a suppository, or a tablet.

The physician and patient have every right to expect that the stated amount of medicament is, in fact, present in each and every dose product and that the acceptable variations of amount fall

TABLE II.—RELATION BETWEEN MINIMUM UNIT DOSE AND ANALYTICAL SAMPLE OF SELECTED TABLETS IN U. S. P. XV AND B. P. 1958

Ref.	Medicament	Analytical Limits, %	Scale of Scrutiny, Dose, mg.	Assay Sample: Dose Units Used	Assay Sample: Wt. as Active Principle, mg.	Analytical Sample: Wt. as Active Principle, mg.	Analytical Sample: Equivalent Dose Units
B. P.	Acetomenaphthone	92½–107½	0.5	400	200	200	400.0
B. P.	Ascorbic acid	90–110	25.0	20	500	50	2.0
U. S. P.	Atropine sulfate	90–110	0.3	200	60	60	200.0
B. P.	Carbimazole	90–110	5.0	20	100	6	1.2
U. S. P.	Colchicine	90–110	0.5	50	25	25	50.0
U. S. P.	Cortisone	90–110	25.0	10	250	25	1.0
B. P.	Cortisone	90–110	25.0	20	500	10	0.4
U. S. P.	Digitoxin	90–110	0.1	50	5	2	20.0
U. S. P.	Reserpine	90–110	0.1	20	2	1	10.0
U. S. P.	Stilbestrol	90–110	0.1	100	10	10	100.0

within precise and, preferably stated, overall limits. Mixtures of powders and products derived thereform may, in fact, fail to comply with this requirement. The final analytical control provides the last check to ensure that the product contains the stated dose of medicament, and it follows from medical requirements that the analytical techniques adopted should, and even must be able to, check adequately the magnitude of minimum unit dose and also provide assessment of the possible variations in amount per unit dose due to limitations in manufacturing procedures.

The ideal condition is to be found in a simple random analytical sample, the size of which is smaller than the minimum unit dose, in which the analyst is confident that his analysis truly represents the specification of the bulk of the material and would be identical with that of any other random sample of the same size. In this case it may be unreservedly assumed that any dose unit contains the required amount of medicament. Such a condition is found in medicaments in the form of simple solutions with low viscosities of say less than 10 centipoises. If, however, the size of the analytical sample exceeds the normal minimum dose, uncertainty of the accuracy of the dose must always exist to a lesser or greater extent according to the number of dose units which are used to make up the analytical sample. This is because there is the possibility of variation in content within the units comprising the sample. From a physician's or patient's point of view, these unknown variations will be in addition to those included in the range allowed by the analysis as well as those inherent in measuring the dose unit concerned. These unknown variations will be small or nonexistent in materials which can be adequately mixed but in the case of materials which are difficult such as powders, they can be quite large so that apparently identical unit doses could produce important differences in pharmacological response.

To illustrate this point reference should be made to the official requirements for some products of powders, i. e., tablets, in the U. S. P. XV and B. P. 1958. (See Table II.) The smallest dose in which the medicament is normally prescribed has been selected because it is in this condition that, between doses, the greatest variation is possible. It will be seen that 400 acetomenaphthone B. P. dose units or 200 atropine sulfate U. S. P. dose units are required to produce a minimum analytical sample. Thus the variations to be found between 200 individual doses of atropine sulfate have been averaged to give a mean content of drug with an allowable error of 10% about the stated content. Thus any imperfection of mix in any one tablet will be masked by the contribution made by 199 units which also form the analytical sample. On the other hand only two-fifths of the minimum dose of cortisone is required as the analytical sample for the B. P. assay yet the analyst has been required to take 20 such dose units to make his sample. Other examples speak for themselves. It will be seen that the emphasis has been to obtain a reasonable mean and is probably favorable to the manufacturer. Products only fail to pass the test if there has been a gross error of judgment in manufacture when, correctly, the batch should be rejected.

It has been deemed necessary to check the uniformity of weight of individual products, but no method is given or suggested so that the uniformity of mix may be checked on a minimum dose unit scale. Thus the uniformity of mix of the ingredients is left to the inherent mixing capability of the machine and the integrity of the operator to run the machine to give the optimal conditions of mix if he knows them.

In practice, where the pharmacological intention of the dose is to correct a deficiency or

maintain a medication, as in ascorbic acid or digitoxin, the patient's body probably can accommodate the possible product-to-product variation in dose, but when the analytical sample rises above 50 dose units, the course of treatment must be long before it can be safely assumed the patient has taken a mean dose consistent with the mean content as shown by analysis. When medication depends on the effect produced by only one or two dose units, then it is reasonable to assume that sufficient attention is paid to the checking of individual dose units. It should be a matter of professional pride that this is done. However there are certain principles of probability which come into the process of mixing and these must be clearly borne in mind when a mixing procedure or checking scheme is being developed.

Because there is a trend towards dry mixing of powders for slugging in tablet manufacture and for filling capsules, it is a useful exercise to assess the statistical aspects of obtaining a unit dose with the correct proportion of ingredients from a truly randomized bulk mixture of equal sized particles of ingredients present in their correct proportions. The probability of obtaining a sample containing the correct proportions of the ingredients varies with the number of particles taken for the sample (10). Thus, in taking samples from an infinite supply of a 50:50 mixture of P and Q, a sample of n particles will on an average contain $n/2$ particles of P. But any one sample may contain less than this number with a corresponding increase in Q. The error to be expected can be expressed mathematically in the form given by the standard deviation about the mean, and it can be shown that multiples of the standard deviation may be used to determine confidence limits to give the proportion of samples, consisting of a given number of particles, that would have less than a given variation in the proportion of a selected ingredient.

For example (See Table III), in the case of a 50:50 mixture, the standard deviation of one component, expressed as a percentage of the whole, is given by $100/n$, where n is the number of particles in the sample. This means that if a sample of 100 particles were taken, one could be confident that 68.3 per cent of samples would have less than a 10 per cent error in the proportion of P, and in like manner 95.5 per cent of samples would have less than a 20 per cent error, while 99.7 per cent samples would have less than a 30 per cent error in P. If the sample size n, were raised to 10,000 particles the errors for each confidence level will have reduced to 1, 2, and 3 per cent, respectively, while if n is a million particles, the errors will have been reduced to 0.1, 0.2, and 0.3 per cent of the desired proportion.

If, however, the proportion of one ingredient is small (see Table IV), then the standard deviation from the mean of the proportion of a given ingredient, expressed as a percentage, may be estimated by $100/\sqrt{pn}$, where p is the proportion of the material expressed as a fraction and n is the number of particles in a sample. It will be seen that for a 1 per cent dispersion and a sample of 900 particles, the confidence levels will be as follows: 68.3 per cent samples would have less than 30 per cent error in the proportions of P, 95.5 per cent samples would have less than 60 per cent error in the proportions of P, and 99.7 per cent samples would have less than 90 per cent error in the proportions of P. To assume reasonable errors of 2, 4, and 6 per cent, respectively, the total number of particles per sample must be of the order of 250,000. Applied to dry mixing of powders, this means that for a dose of 1 mg. of drug in a product weighing 100 mg. the total material should be reduced in size to give at least 250,000 particles so that the unit dose, based on a perfect mixing operation alone, may be within 4 per cent of the correct dose for 95.5 per cent of the products and within 6 per cent for 99.7 per cent of the products. Assuming that the drug and diluent have a specific gravity of 1.5, the mean particle size of all ingredients must be less than 50 μ.

One of the smallest doses in either pharmacopeia is 0.1 mg. Assuming that this is to be presented as a 40-mg. dose unit, the dilution is 1 in 400. Working to the same confidence

TABLE III.—PERFECT MIXING[a]

Confidence Level of No. of Samples	% Limiting Error in p: n = 100	n = 10,000	n = 1,000,000
68.3	10	1	0.1
95.5	20	2	0.2
99.7	30	3	0.3

[a] Proportion of $P:Q$ = 50:50; no. of particles in a sample n; standard deviation of sample, % = $100/\sqrt{n}$.

TABLE IV.—PERFECT MIXING[a]

Confidence Level of No. of Samples, %	% Limiting Error in p: p 0.01, n 900	0.01, 250,000	0.025, 1,000,000
68.3	30	2	2
95.5	60	4	4
99.7	90	6	6

[a] Proportion of $P:Q$ = p:1-p; no. in sample = n; standard deviation of sample, % = $100/\sqrt{pn}$.

limits as the preceding example, the unit sample should contain at least 1 million particles so that the mean particle size should be less than 4 μ.

It must be emphasized that the foregoing calculations have been based on a condition of perfect mixing. If this is not obtained, the variation in behavior between unit doses is subject to additional errors. It is clear that the operation of powder processing is by no means understood or worked out and that the worst enemy in this field is probably complacency through ignorance. Pharmaceutically it must always be the aim to supply precise individual dose units, but it must be concluded that present day practices in powder technology sometimes fall short of this attainment.

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Studies on *Morinda citrifolia* L. II*

By HEBER W. YOUNGKEN, Sr., HOWARD J. JENKINS, and CALVIN L. BUTLER†

The results of a study of the anatomy of the stem of *Morinda citrifolia* L. are described. A preliminary report of the pharmacodynamic study of a 95 per cent alcoholic extract of the root on the isolated uterine horn of an albino rat showed some measure of sedation using doses of 1 to 5,000 to 1 to 36,000. A water decoction of the crude whole root, injected intravenously in the anesthetized dog, in a dose representing 4 Gm. produced a marked lowering of blood pressure which lasted approximately twenty-six minutes. It appears that the hypotensor activity resides in the water-soluble fraction of the root of *Morinda citrifolia*. The results of *in vivo* experiments gave evidence of a low order of toxicity of *Morinda citrifolia* root.

AN INVESTIGATION of the root of *Morinda citrifolia* L. and of herbarium material of the same species was reported in a previous paper (1). The studies reported here include an investigation of the anatomy of the stem of *M. citrifolia*. *In vitro* tests on segments of the anterior horn of the albino rat uterus were carried out with an alcoholic extract of the root, and the effects of intravenously injected alcoholic and aqueous extracts of the root were observed for hypotensor action in rabbits and dogs.

PREPARATION OF MATERIALS[1]

The materials were prepared for anatomical studies by repeatedly boiling and sectioning stem segments approximately 5, 12, and 18 mm. in diameter. The bark portions were placed between the flat surfaces of cork prior to sectioning by the sliding microtome and by free hand. Transverse, radial longitudinal, and tangential longitudinal sections were cut and mounted separately in water, dilute glycerin, 2% potassium hydroxide solution, iodine water, and phloroglucin-HCl. Schulze's maceration method was employed to facilitate the separation of the lignified from the nonlignified elements, followed by teasing them apart on the microslide and mounting for microscopic study. Structures were studied under the low and high magnifications of the microscope. Scrapings of representative parts of the branch were also examined microscopically in these reagents and the results recorded. Starch grains were examined and measured in water mounts of scrapings and powdered stem material.

ANATOMY OF THE STEM OF *Morinda citrifolia* L.

Examination of transverse sections 5 mm. in diameter revealed the following structure: (*a*) an epidermis of clear cells with strongly cutinized outer and radial walls; (*b*) a hypodermis of two zones, the outer of which was composed of a layer of parenchyma, the inner zone consisting of two layers, the outer of an almost continuous band of stone cells interspersed here and there with groups of sclerenchyma fibers, the inner layer

* Received August 21, 1959, from the Massachusetts College of Pharmacy, Boston.
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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
[1] The materials used in these studies consisted of an entire long branch of *M. citrifolia* collected from a plant growing in the Indian Museum Garden, Calcutta, India, by Dr. Sunil C. Datta; and about 20 pounds of roots and 20 pounds of stems of *M. citrifolia* gathered in India by collectors of the S. B. Penick Co.

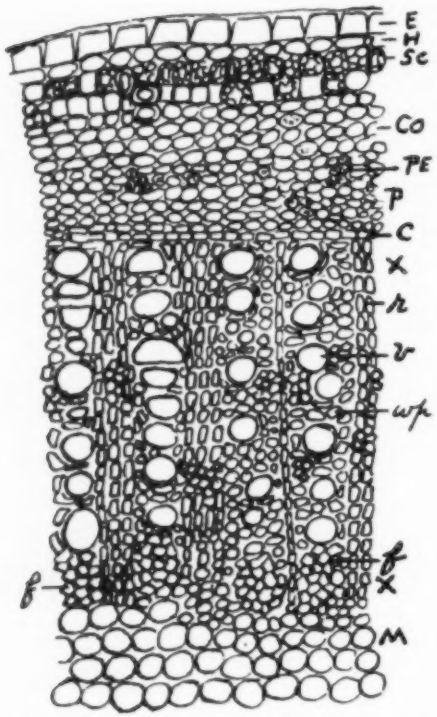

Fig. 1.—Representative portion of a transverse section of a distal segment of a stem 5 mm. in diameter of *Morinda citrifolia L.* *E*, Epidermis; *H*, hypodermis containing stone cells and groups of fibers (Scl); *Co*, cortex; *Pe*, pericycle; *P*, phloem; C, cambium; *X*, xylem; *r*, xylem ray; *v*, vessel; *wp*, wood parenchyma, *f*, wood fiber; *M*, pith. Magnified.

consisting of parenchyma and scattered stone cells with an occasional small group of sclerenchyma fibers; (*c*) a cortex consisting of four layers of cortical parenchyma some of the cells of which contained small starch grains; (*d*) a pericycle of about three layers and consisting chiefly of ordinary parenchyma in which groups of pericyclic fibers were scattered; (*e*) a narrow phloem of sieve elements and phloem parenchyma traversed by phloem rays; (*f*) a wavy cambium of meristematic cells; (*g*) a broad xylem of numerous wood wedges separated from each other by xylem rays from 1 to 3 cells wide. The walls of the latter were pitted, nonlignified to lignified. The wood wedges contain vessels which are mostly isolated and also arranged radially, numerous tracheids and groups of wood fibers as well as considerable apotracheal wood parenchyma with pitted walls. Prominent groups of wood fibers with strongly lignified walls appeared especially abundant near the inner margin of the wedges. The xylem rays were composed mostly of radially elongated cells with nonlignified to lignified walls. They were interrupted in places by groups of fibers with lignified walls; (*h*) a pith composed of rounded pith parenchyma with cellulose walls which showed pitting. This region already showed evidence of centrifugal disintegration as was shown by its hollow center. See Fig. 1.

Longitudinal radial sections showed axially arranged raphide sacs containing acicular crystals of calcium oxalate up to 100 μ in length, occurring in both cortex and phloem, spiral, simple pitted, and bordered pitted vessels and tracheids with lignified walls, elongated wood parenchyma cells with lignified pitted walls, and thick- and thin-walled wood fibers with oblique pits in their lignified walls.

Transverse sections of the stem from 8 to 18 mm. in diameter were characterized by a progressive increase in the number of sclerenchyma elements in the hypodermis and pericycle, by the occurrence of scattered groups of fibers in the phloem, an increased lignification of the walls of the xylem elements, and an increased disintegration of the pith in centrifugal fashion. See Fig. 2.

The vessels were isolated and in small groups of 2 to 4 and in radial groups of 2 to 10. In longitudinal sections of the same stem diameters, their ends were oblique, straight, or rounded and exhibited large circular openings in their perforation plates. They measured up to 684 μ in length and up to 114 μ in width.

Longitudinal radial and tangential sections of the stem, 8 to 18 mm. in diameter, showed a progressive decrease in the number of raphide sacs and an

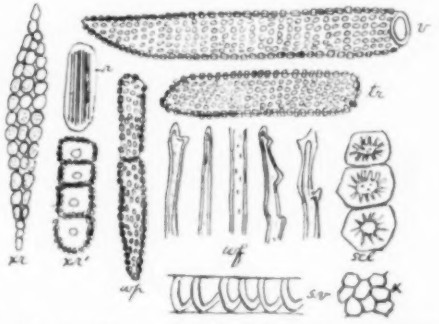

Fig. 2.—Diagnostical histological elements occurring in the stem of *Morinda citrifolia* L. *xr*, xylem ray as observed in a tangential section, *xr'*, 4 cells of a xylem ray as observed in a transverse section; *r*, raphide sac containing acicular crystals of calcium oxalate; *wp*, wood parenchyma; *v*, pitted vessel; *tr*, pitted tracheid, *sv*, spiral vessel; *wf*, wood fibers; *scl*, stone cells; *K*, cork.

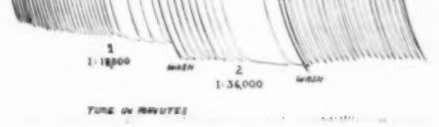

Fig. 3.—Effect of *Morinda citrifolia* root 95% alcohol Soxhlet extract on the right horn, isolated uterus of an anestrus rat, *in vitro*.

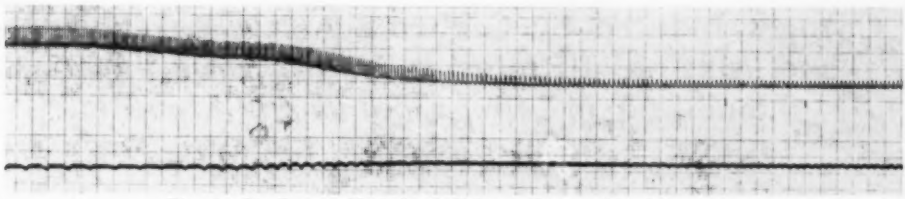

Fig. 4.—Studies on *Morinda citrifolia;* effect on blood pressure.

increase in the number of stone cells in the bark. The tracheids were of the same types as the vessels but mostly shorter and always with closed ends. The wood fibers were straight to curved, a number showing lobing or dentation toward one or both ends. Their ends were mostly acute or attenuate, their walls strongly to moderately lignified with oblique pits, and their lumina irregular in width. They measured up to 1,400 μ in length. A number of the parenchyma cells possessed globules of oil. The starch grains were fewer than those previously observed in the root, and mostly single. They measured up to 15.2 μ in diameter.

PRELIMINARY *IN VITRO* PHARMACODYNAMIC STUDY

Preliminary *in vitro* pharmacodynamic experiments upon isolated tissues were made in accordance with the Magnus method as described by Burn (2). Segments of the isolated uterine horn of the albino rat were employed as the test tissue, and used by preference since results were more easily producible. Further, the various stages of the estrus cycle can be easily determined by examination of the vaginal smear before sacrifice. In our hands it was found that the uterine tissue of rats at anestrus responds much more uniformly than at other times of the cycle.

The apparatus used was of the general type and consisted of a constant temperature bath automatically maintained at 37.5°, containing a 100-cc. muscle chamber in which a horn or a strip of the uterus, freed from its mesenteric adherences, was immersed. Oxygenation of the tissue was assured by using a tank of oxygen equipped with a valve which allowed a regular and constant flow of the gas to bubble through the nutrient solution. The nutrient solution was of the following formula:

NaCl (reagent)	9.0 Gm.
KCl (reagent)	0.42 Gm.
$CaCl_2$ (reagent) (anhydrous)	0.24 Gm.
$NaHCO_3$ (reagent)	0.5 Gm.
Dextrose	0.5 Gm.
Distilled H_2O	q. s. 1000.0 ml.

It was found that the addition or omission of 2.5 mg. magnesium chloride to this formula makes little difference in the response of the isolated organ and, accordingly, it was omitted in this series of experiments.

A series of ten experiments using a 95% alcoholic extract of *Morinda citrifolia* root was made. It was not found necessary to dealcoholize the test substance since the small volumes of alcohol involved had been shown previously to have no effect on uterine tissue.

Figure 3 illustrates the typical response we observed in the majority of the experiments.

At (I) a dilution of 1:18,000 of a 95% alcoholic extract was injected into the muscle chamber. A characteristic decrease in activity as witnessed by a decrease in amplitude and rate of contractions with some lowering of tone was shown. Upon washing several times with fresh nutrient solution normal activity was resumed.

At (II) a dilution of 1:36,000 showed still more sedation than the first dose which indicates that the tissue must have been somewhat conditioned to the drug. Normal activity occurred upon washing.

In all the experiments some measure of sedation was shown, using doses ranging from 1 to 5,000 to 1 to 36,000.

THE PRESSOR ACTIVITY OF SELECTED FRACTIONS OF THE POWDERED WHOLE ROOT OF *Morinda citrifolia*

Alcohol-Soluble Portion.—Twenty grams of *Morinda citrifolia* root was exhausted with 250 ml. chloroform in a Soxhlet apparatus for ten hours. The marc was extracted with 250 ml. of 70% ethanol. The ethanol extract was dried under reduced pressure and the orange-red residue was redissolved in 60% propylene glycol. This preparation, injected intravenously into the rabbit in a dose representing 400 mg. of the crude root, failed to bring about a significant lowering of blood pressure.

Water-Soluble Portion.—A water decoction of the crude whole root was made by boiling the drug for ten minutes. The decoction was then strained and filtered. The cloudy filtrate obtained was not filtered further. This preparation injected intravenously in the anesthetized dog in a dose representing 4 Gm. of the crude whole root produced a marked lowering of blood pressure which lasted approximately twenty-six minutes, see Fig. 4. This pharmacologic test supports Dr. Van Ho's clinical observations on the hypotensor action of the drug (3).

Time, min.	Blood Pressure	Heart Rate
pre-drug	165/95	150
1	135/80	222
2	20/0	108
3	40/15	108
5	110/70	102
9.5	160/105	84
23.5	150/100	114
26	165/95	130

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Changes Induced by Gibberellic Acid on Growth and Alkaloid Patterns in *Datura stramonium* Linné and in *Atropa belladonna* Linné*

By ROBERT E. BRUMMETT and LEO A. SCIUCHETTI

Stramonium and belladonna were administered single 100-mcg. doses of gibberellic acid by application to the uppermost leaves of the plants. Growth and alkaloid patterns following treatment are described. A modified assay procedure for 25-mg. samples of material is outlined. The total plant alkaloids in treated stramonium were slightly less than controls at each harvest period. Total root alkaloid production, however, was increased about 50 per cent. Interesting differences in fresh and dry weights were induced in belladonna at different stages of maturity. Younger seedlings indicated significantly decreased growth, whereas older plants demonstrated significant increases. Due to increased growth the older plants displayed about a 32 per cent increase in total alkaloid production. The age of the plant when treated appeared to be an important factor in causing increased alkaloid production.

PREVIOUS RESEARCH (1) has demonstrated that gibberellic acid (G. A.) when administered in the form of an aqueous spray in concentrations of 100 and 1,000 p. p. m. to the leaves and tops of *Datura stramonium* and *Atropa belladonna* produced a favorable effect on the growth of the former, but a less desirable response on the latter. Decreased concentrations of alkaloids were generally noted in the morphological parts of treated plants. Similar trends have been reported in *Hyoscyamus niger* (2) and in different *Nicotiana* species (3).

This study differed from our previous work (1) in that a single 100-mcg. dose of G. A. was administered to the uppermost leaves of the plants, patterns of growth and alkaloid formation were determined from periodic harvests of plant parts rather than from a terminal harvest, and, with belladonna, it was desired to determine whether the age of the plant (more appropriately, the stage of plant development when treated) would be an important factor in the response to the gibberellin effect.

EXPERIMENTAL

Procedure.—*Datura stramonium* plants employed in this study were grown under greenhouse conditions in a previously fertilized[1] plot of ground. Seeds were germinated in culture flats containing soil composed of two parts each of sand and sandy loam, and one part of peat moss. On June 24, 1958, 56 twenty-two-day-old plants were transplanted into the greenhouse soil plot. These were planted in three rows which were two feet apart and the plants were spaced two feet apart in each row. The plants were then divided into seven groups of eight each and designated as controls and plants to be treated. On July 1, 1958 (zero-time), the 24 plants that had been designated for treatment were given a single dose of 100 mcg. of G. A.[2] This quantity was delivered from a micropipet onto the surface of the youngest unfolding leaf by applying 0.02-ml. portions of a freshly prepared aqueous solution (containing 5 mg. of G. A. per ml.). Height measurements were taken twice weekly. The plan for harvesting stramonium was as follows: 32 control plants were divided into groups of eight each and each group was harvested at zero, one, two, and three weeks; 24 treated plants were similarly divided and harvested at one-, two-, and three-week intervals. Division of the plant into its morphological parts, fresh and dry weight determinations, pulverization, and storage of the powdered material were conducted in a manner described in a previous publication (1).

The belladonna seeds were germinated in a manner similar to that for stramonium. The average germination date for the first planting was April 17, 1958. This planting is hereafter designated as the older plants. The average germination date for the second planting was June 1 (hereafter designated as the younger plants). On June 26, 1958, 80 plants consisting of 40 seedlings from each planting were transplanted into a fertilized soil plot in the greenhouse. Treatment time was July 3, 1958. The younger plants (A series) were thirty-three-day-old seedlings at the start of the experiment; the older plants (B series) were eleven-week-old seedlings. The control group within each series consisted of 24 plants, from which eight each were selected at random and harvested at zero, three, and six weeks. The treated group consisted of 16 plants from which eight each were harvested at three and six weeks. At zero time the treated plants

* Received August 21, 1959, from Oregon State College, School of Pharmacy, Corvallis.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
The work with stramonium was done by Robert E. Brummett and the manuscript resulting from this research was selected as the 1959 Edwin Leigh Newcomb Award.
The study with belladonna was done by Leo A. Sciuchetti and was supported in part by a grant from the General Research Fund of the Graduate School of Oregon State College.

[1] Each plant was furnished with 100 Gm. of organic fertilizer (analysis—5% total nitrogen, 3% available phosphate, 2% available potash) that had been worked into the soil prior to planting.

[2] The gibberellic acid used in this study was furnished through the courtesy of Dr. Edwin F. Alder, Agricultural Research Center, Eli Lilly and Co., Greenfield, Ind.

TABLE I.—WEIGHTS OF BELLADONNA PLANT PARTS FOLLOWING A SINGLE TREATMENT WITH GIBBERELLIC ACID (AV./PLANT/GROUP)

Sample and Harvest Time, wk.	Total Weight			Leaves-Tops			Stems			Roots		
	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %
A Series												
Control, 0	0.130	0.012	...	0.100	0.009	...	[a]	[a]	...	0.030	0.003	...
Control, 3	3.64	0.304	...	3.31	0.25	...	[a]	[a]	...	0.33	0.054	...
Treated, 3	2.81	0.248	82	2.69	0.21	84	[a]	[a]	[a]	0.22	0.038	70
Control, 6	44.93	4.59	...	35.67	3.74	...	5.32	0.38	...	3.94	0.47	...
Treated, 6	26.26	3.18	69	19.47	2.41	64	3.91	0.40	105	2.88	0.37	79
B Series												
Control, 0	0.486	0.052	...	0.450	0.047	...	[a]	[a]	...	0.036	0.005	...
Control, 3	9.65	0.821	...	8.29	0.670	...	[a]	[a]	...	1.36	0.151	...
Treated, 3	13.11	1.25	152	11.34	1.05	157	[a]	[a]	[a]	1.77	0.199	133
Control, 6	111.35	11.07	...	82.03	8.51	...	17.66	1.42	...	11.66	1.14	...
Treated, 6	169.34	16.30	148	112.52	11.24	132	38.08	2.92	206	18.74	2.14	188

[a] No differentiation into stem portions so shoots were designated as leaves-tops. A series plants were thirty-three-day-old seedlings at 0 time; B series, eleven-week-old plants.

were administered 100 mcg. of G. A. in the manner previously described. Height measurements were taken twice weekly. At zero time and three weeks each plant was divided into shoot (labeled leaves-tops) and root portions. At this stage of development stem growth was almost negligible and the small amount available was combined with the leaves and tops. By the sixth week, however, stem development was satisfactory so that this additional portion was collected. Otherwise, the harvesting procedure was identical with that described for stramonium.

Growth Effects.—The treated plants generally demonstrated the gibberellin effects previously reported by Smith and Sciuchetti (1). The proneness of treated plants to insect attacks was not noted in stramonium and only to a slight extent in belladonna. This may have been due to the fact that the G. A. was placed on one leaf only in this experiment instead of being sprayed onto the aerial parts of the plant, the soil, and the plant container. The insects may have been attracted to the G. A. itself rather than by any reduced resistance in the plant.

The height increases in both plants were not as great as that induced by a spray treatment (1). With belladonna the maximum increases were attained at the fourth week; an 80% increase was noted in the younger plants and a 106% increase in the older plants. The rapidity of response and the sensitivity to this growth effect were more pronounced and of longer duration in the older plants. Bukovac and Wittwer (4) have also reported that the age of bean plants influenced their response.

Fresh and Dry Weights.—A favorable response on growth was generally induced by the G. A. treatment in stramonium as indicated by fresh and dry weight data. The growth rate was more rapid than that previously reported (1). Further, the roots demonstrated a beneficial effect (up to an 80% increase in root dry weight at the second harvest) contrasted with the decreased growth reported in earlier work (1). Two factors may have contributed to increased root growth: the plants in this experiment were grown directly in the soil instead of pots, thus, "root-bounding" was not a possibility; and the plants received fertilizer. Other workers (5, 6) have indicated that fertilization is an important factor in order to induce a favorable growth response from treatment with G. A.

With belladonna the younger plants (A series) demonstrated generally significant reductions in fresh and dry weights, whereas the older plants (B series) indicated significantly increased weights (Table I). The age of the plant (stage of plant development) appeared to be an important factor in obtaining a beneficial response from the treatment.

Analysis for Alkaloids.—The dried plant parts, using pooled samples, were assayed for total alkaloids by a new procedure which was found to be efficient and accurate for 25-mg. samples of material. The method employed the citric acid buffer described by Witt and Youngken (7) for the extraction of the powdered drug and an extractor as described by French and Gibson (8). The extraction procedure was as follows: A small cotton plug was placed in the bottom of the extractor. The 25-mg. sample was then placed in the extractor and shaken into a compact column. Another plug was placed gently on the top. Citric acid buffer (0.4 ml.) was placed on the plant material and allowed to macerate for two hours. The aqueous solution containing the extractive was withdrawn directly into a 25-ml. separatory funnel containing 15 ml. of chloroform. First, a 3-ml. portion of cirtric acid buffer, and then, two 1-ml. portions of buffer were used to complete the extraction and rinse the extractor. The combined collection of the aqueous phases was made basic to litmus paper by using 28% ammonium hydroxide. The contents of the separatory funnel was then shaken by hand for one minute and allowed to stand overnight. The 15-ml. portion of chloroform was then removed and collected in airtight glass containers. The remaining phase was shaken for one minute, using an additional 10-ml. quantity of chloroform. This was then added to the first 15-ml. portion. Ten-milliliter aliquots of the combined 25-ml. sample were then analyzed for total alkaloids by the Vitali-Morin reaction.

Significant decreases in the concentration of alkaloids (calculated as scopolamine) were induced by the treatment in the leaves-tops and stems of stramonium while slight changes were observed in the roots (Fig. 1). The largest decreases per plant part which were noted were a 30% reduction in the leaves-tops at the third week and a 55% reduction in the stems at the second week.

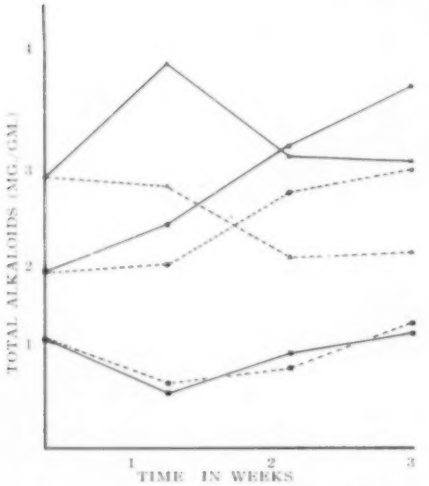

Fig. 1.—Alkaloid pattern in plant parts of stramonium, expressed as scopolamine. ●——●, Control leaves; ●····●, treated leaves; ▲——▲, control stems; ▲····▲, treated stems; ■——■, control roots; ■····■, treated roots.

The G. A. induced a similar effect in belladonna since reduced concentrations of alkaloids (expressed as hyoscyamine) were noted in the parts of the treated plants of each series (Fig. 2). The decreases were greater in the younger plants than in the older plants. Decreased alkaloid concentrations resulting from a G. A. treatment have also been reported by others (1–3) in members of the solanaceae.

Total Plant Alkaloids.—The total alkaloids per plant and per plant organ were obtained by multiplying the dry weight of the plant organ by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in milligrams (Table II). Slight decreases in the total alkaloid content per plant were induced in stramonium by the treatments. The roots of treated plants, however, demonstrated increases of 40% and 54% for the second and third harvests, respectively. This was due mainly to a significant increase in root growth.

The treatments resulted in diverse effects on the total alkaloid content of belladonna. The younger plants (A series) produced about 40% less alkaloids at the sixth week compared with controls, whereas the older plants (B series) displayed about a 32% increase in the total alkaloid content (Table II). The significantly increased alkaloid production in the older plants was due primarily to the favorable effect of G. A. in promoting more extensive growth. The age of the plant when treated appeared to be an important factor in causing increased growth which, in turn, resulted in increased alkaloid production per plant.

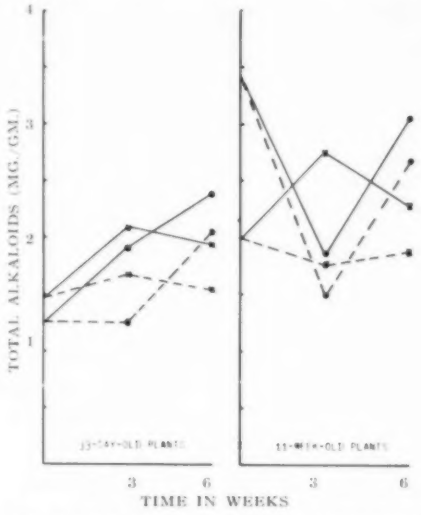

Fig. 2.—Alkaloid pattern in belladonna organs, expressed as hyoscyamine. ●, Shoots; x, roots; ——, controls; - - - -, treated.

TABLE II.—TOTAL ALKALOID CONTENT OF STRAMONIUM[a] AND BELLADONNA[b]

Sample and Harvest Time, wk.	—Total Alkaloid Content,[c] mg.— Per Plant	Leaves and Tops	Stems	Roots
Stramonium				
Control, 0	0.06	0.04	0.02	0.01
Control, 1	0.36	0.25	0.11	0.03
Treated, 1	0.31	0.20	0.08	0.03
Control, 2	2.06	1.71	0.30	0.05
Treated, 2	1.98	1.58	0.33	0.07
Control, 3	12.94	11.17	1.42	0.35
Treated, 3	11.15	8.68	1.93	0.54
Belladonna				
A Series				
Control, 0	0.015	0.011	..	0.004
Control, 3	0.590	0.478	..	0.112
Treated, 3	0.324	0.260	..	0.064
Control, 6	10.70	8.49	1.36	0.851
Treated, 6	6.31	4.94	0.80	0.574
B Series				
Control, 0	0.170	0.160	..	0.010
Control, 3	1.67	1.25	..	0.415
Treated, 3	1.94	1.58	..	0.355
Control, 6	32.74	25.16	4.98	2.60
Treated, 6	43.23	31.47	7.74	4.02

[a] Total alkaloids expressed as scopolamine.
[b] Total alkaloids calculated as hyoscyamine.
[c] Alkaloid content for plant parts calculated from dry weight and alkaloid analyses data; total plant alkaloid = leaves and tops + stems and roots; based on av./plant/group.

SUMMARY

1. A modified assay procedure for the estimation of total alkaloids in 25-mg. samples of powdered drug is outlined.

2. Characteristic gibberellin effects were induced in stramonium and belladonna by the treatments. In the latter, height measurements indicated that older plants reacted more rapidly and for a longer period of time to this response than did younger plants.

3. Slight changes in the total dry weight of stramonium were induced, but root growth was increased up to 80 per cent. In belladonna the total dry weight of the younger plants was reduced about 30% while that of the older plants was increased about 48%.

4. The concentration of alkaloids in the organs of each species was generally reduced. Total alkaloid production in treated stramonium was somewhat less than that in controls, but total root alkaloids were increased about 50 per cent.

5. At the sixth week following treatment younger belladonna plants produced about 40 per cent less total alkaloids than controls, whereas older plants displayed about a 30 per cent increase compared with controls.

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Digitalis X. The Infrared Absorption Spectra of Some Digitalis Glycosides and Aglycones*

By FREDERICK K. BELL

The infrared absorption spectra of commercial samples of the three lanatosides, their aglycones and their three intermediate glycosides, digitoxin, gitoxin, and digoxin, have been scanned between 2.5μ and 15μ using the potassium bromide disk technique. Some of the more pronounced characteristics of these spectra have been noted and their possible significance in the identification and differentiation of these highly important cardiotonic substances has been indicated.

WITH THE RAPIDLY increasing availability of the technique of infrared absorption spectrometry it seems timely that an examination of the infrared absorption spectra of some of the more common digitalis principles should be undertaken. The results of such a study could be reasonably expected to provide useful information for those interested in the isolation, purification, and identification of these highly important glycosides. It also seems probable that because of its unusual scope, the infrared absorption spectrum will assume as increasingly important position along with other well-known physical constants in purity and identification rubrics. In the extensive studies of the infrared absorption of steroids (1) only one of these digitalis substances, digitoxigenin, and several derivatives have been reported.

For this preliminary study the three lanatosides and some of their degradation products have been selected for examination. This selection was based on several considerations. Commercial availability and also the inclusion of some of the most important cardiotonic agents in clinical use were of prime importance, as well as the interrelationships of the chemical structures of the various compounds.

EXPERIMENTAL

The substances examined together with the source of supply are as follows: lanatosides A and B (Sandoz Pharmaceuticals), lanatoside C (N. F. Reference Standard), digitoxin (U. S. P. Ref-

* Received August 1, 1959, from the School of Medicine, University of Maryland, Baltimore 1, Md.
The author gratefully acknowledges the kind interest and suggestions of Dr. Leopold May of the Psychiatric Institute.

erence Standard), gitoxin (Hoffmann-LaRoche and Co.), digoxin (U. S. P. Reference Standard), digitoxigenin (Sterling-Winthrop Research Institute), gitoxigenin (Sterling-Winthrop Research Institute), and digoxigenin (Burroughs Wellcome and Co.). Digitoxose (Hoffmann-LaRoche and Co.) was also examined. Each substance was used as received in its original container and for this preliminary study it seemed desirable, particularly with respect to the practical significance of the data obtained, to postpone the purification procedures which will be required ultimately.

The Perkin-Elmer model 137 infracord was used for scanning the absorption spectra between 2.5μ and 15μ, and the potassium bromide disk technique was employed for the preparation of the samples. Potassium bromide, infrared quality, was obtained from the Harshaw Chemical Co. For each glycosidal substance, disks were prepared on the basis of 2 mg. of substance to 300 mg. of potassium bromide. In the case of digitoxose, this ratio was 1 mg. to 300 mg. To the weighed amount of substance, which was transferred to an agate mortar, was added a small portion of the 300 mg. of potassium bromide. After this mixture was thoroughly ground by hand, the balance of the potassium bromide was added, and the final treatment was directed toward thorough mixing. The final mixture was transferred to a die (No. H-920, Hilger and Watts Ltd.) which was connected to a Cenco Hyvac pump. After a period of three minutes of evacuation, the die was compressed with a laboratory model Carver hydraulic press for a period of one minute at its maximum pressure (20,000 pounds load). The disk thickness was approximately 1 mm. In the preparation of the disks every effort was made to standardize the technique on a qualitative and quantitative basis so that the comparative value of the results could be maintained at as high a level as possible. Several disks were prepared for each substance and their spectra were scanned. Typical absorption spectra are shown in Figs. 1 to 4.

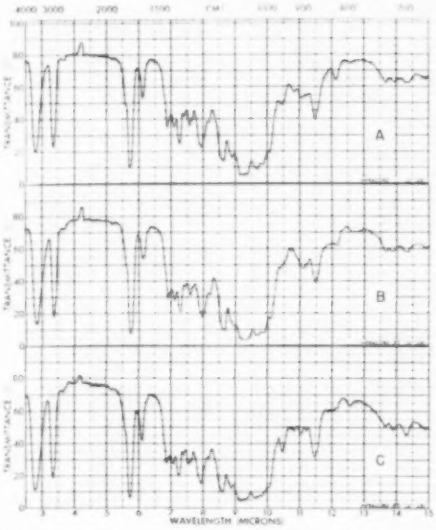


Fig. 1.—*A*, lanatoside A; *B*, lanatoside B; *C*, lanatoside C.

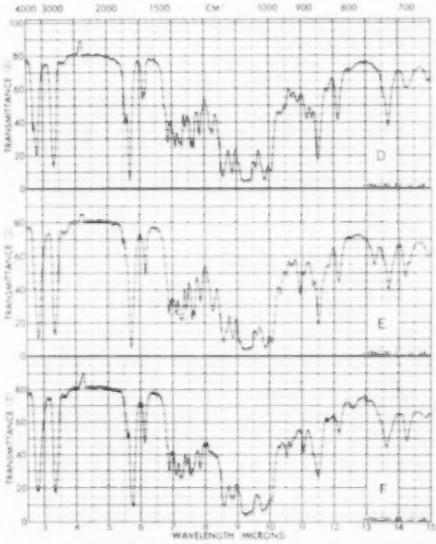


Fig. 2.—*D*, digitoxin; *E*, gitoxin; *F*, digoxin.

DISCUSSION

The structural interrelationships of the digitalis compounds examined are shown in Table I. The carbohydrate moiety of the lanatosides is terminated by the glucose molecule which is attached to an acetylated digitoxose molecule preceded by two additional digitoxose molecules. The absorption spectra of the lanatosides which contain the complete sugar moiety and therefore have the largest molecular weight are shown in Fig. 1. These three absorption spectra are strikingly similar both quantitatively and qualitatively. This is particularly true in the spectra of lanatosides A and B in which the only structural difference (Table I) consists of the presence of an additional hydroxyl group at C 16 in the lanatoside B molecule. The structure of the lanatoside C molecule is characterized by the presence of an hydroxyl group at a new position at C 12. All three spectra display a high degree of absorption in the region between 9μ and 10μ.

Deacetylation and removal of the glucose molecule from the lanatosides result in the formation of the glycosides: digitoxin, gitoxin, and digoxin. These spectra, as shown in Fig. 2, reveal similar absorption patterns in the longer wavelengths at 12.1μ and 14.2μ, and also at 11.5μ and 13.7μ, at which points the absorption of digitoxose (Fig. 4) at 11.4μ and 13.8μ may be a contributing factor. The region of high absorption of the lanatosides between 9μ and 10μ is retained and perhaps extended somewhat toward the shorter wavelengths. It is worthy of mention that while gitoxin and digoxin show a very sharp absorption of medium intensity in the region of 6.1μ, in the spectrum of digitoxin, two bands appear. We have examined five other

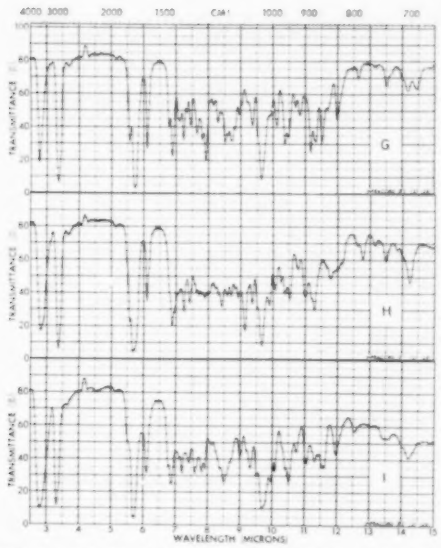


Fig. 3.—*G*, digitoxigenin; *H*, gitoxigenin; *I*, digoxigenin.

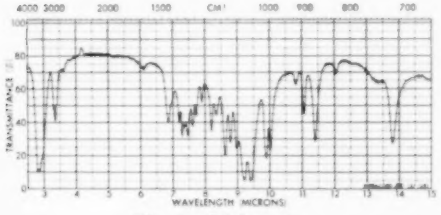


Fig. 4.—Digitoxose.

samples of this glycoside and have found, consistently, either a resolution of two bands or a single band definitely broader than that shown by gitoxin and digoxin.

The absorption spectra of the three aglycones (digitoxigenin, gitoxigenin, and digoxigenin) are shown in Fig. 3. With the complete removal of the sugar moiety there is a marked change in the resolution and transparency in the region between 9μ and 10μ, indicating again the possible influence of the presence of digitoxose in the glycosides on the spectra in this region. The sharp absorption at 6.1μ appears to reach a maximum value in these three genins.

CONCLUSIONS

Although the question of the purity of the substances examined in this study is unanswered, there appear to be a number of conclusions which may be drawn, at least tentatively, and some of these may have some practical significance. There is a striking similarity, both qualitatively and quantitatively, in all nine spectra of the glycosidal substances in the shorter wavelength region below 7μ in spite of pronounced differences in molecular weight (Table I). Since all of the disks of these substances were prepared on the same weight basis, this is probably attributable to the diminution of the comulative effect of the well known absorbing vibrations in this region accompanying the decrease in molecular weight.

It is clear that the experimental method and instrumentation employed are not satisfactory for the identification of the three lanatosides, particularly lanatosides A and B. There is a definite indication, however, that a closer study of the region between 10.5μ and 11.5μ might be fruitful in this connection.

On passing to the absorption spectra of the intermediate glycosides (digitoxin, gitoxin, and digoxin) minor differences appear which may be suitable for purposes of identification. It seems probable that with the diminution of the carbohydrate moiety of the lanatosides, the positions and the number of hydroxyl groups in the steroid nucleus of these substances assume a greater influence on the absorption pattern. Once again, the region between 10.5μ and 11.5μ appears worthy of further study. The absorption of these substances in the region between 13μ and 15μ contrasts markedly with that of the lanatosides and, to a lesser degree, with that of the aglycones.

In the absorption spectra of the aglycones numerous minor differences appear which may be useful for analytical purposes. With these substances, consisting of the steroid nucleus alone, the number and positions of the hydroxyl groups would be expected to exert their maximum influence on the absorption which is in accord with the experimental findings.

TABLE I.—THE STRUCTURAL INTERRELATIONSHIPS OF THE DIGITALIS COMPOUNDS EXAMINED

Substance	Mol. Wt.	Aglycone	$(Digitoxose)_3$	Acetyl	Glucose	OH Groups
Lanatoside A	969.1	Digitoxigenin	+	+	+	C_{14}
Lanatoside B	985.1	Gitoxigenin	+	+	+	$C_{14}C_{16}$
Lanatoside C	985.1	Digoxigenin	+	+	+	$C_{12}C_{14}$
Digitoxin	764.9	Digitoxigenin	+	...	...	C_{14}
Gitoxin	780.9	Gitoxigenin	+	...	...	$C_{14}C_{16}$
Digoxin	780.9	Digoxigenin	+	...	...	$C_{12}C_{14}$
Digitoxigenin	374.5	....	...	...	...	C_3C_{14}
Gitoxigenin	390.5	....	...	...	...	$C_3C_{14}C_{16}$
Digoxigenin	390.5	....	...	...	...	$C_3C_{12}C_{14}$

The nine absorption spectra presented in this study indicate clearly that the three lanatosides can be readily differentiated from the corresponding intermediate glycosides (digitoxin, gitoxin, and digoxin) and from their aglycones by the infrared method. Furthermore, the aglycones can likewise be readily differentiated from the intermediate glycosides and from the lanatosides. Although there are a number of satisfactory chemical tests for the identification of carbohydrates, there may be instances in which the infrared technique may be preferred, especially since the test sample is not necessarily destroyed. The results also show that the three Reference Standard glycosides (lanatoside C, digitoxin, and digoxin) can be differentiated by their infrared absorption.

REFERENCE

(1) Dobriner, K., Katzenellenbogen, E. R., and Jones, R. N., "Infrared Absorption Spectra of Steroids," Interscience Publishers, Inc., New York, N. Y., 1953.

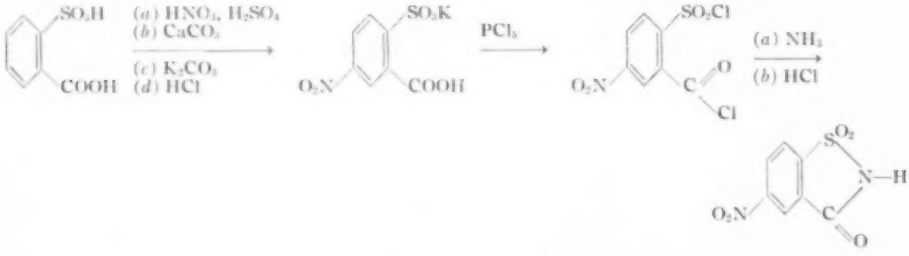
Saccharin Derivatives II*

Synthesis of 4-Nitrosaccharin and Related Compounds

By GLENN H. HAMOR

Five new saccharin derivatives were prepared: 4-nitrosaccharin, 4-aminosaccharin, 4-acetamidosaccharin, 2-ethyl-4-nitrosaccharin, and 2-*n*-propyl-4-nitrosaccharin. Attempts to prepare 5-nitrosaccharin, using procedures described in the literature, gave instead a mixture of two nitrosaccharins, which was shown to consist of the known 6-nitrosaccharin plus 4-nitrosaccharin identical to the above compound. The new saccharin derivatives were tasteless, with the exception of 4-aminosaccharin which had a slightly sweet taste.

IN A RECENT STUDY of saccharin derivatives, the preparation of various nitrosaccharins and related compounds was desired (1). 6-Nitrosaccharin was synthesized by the method of Noyes (2). Only two references have been noted in the literature in regard to saccharins containing nitro or amino groups in positions other than the 6-position. Both of these concerned the 5-position. Stubbs in 1913 (work done in 1892) reported the synthesis of 5-nitrosaccharin (3). This compound was prepared by the nitration of *o*-sulfobenzoic acid by the following series of reactions:



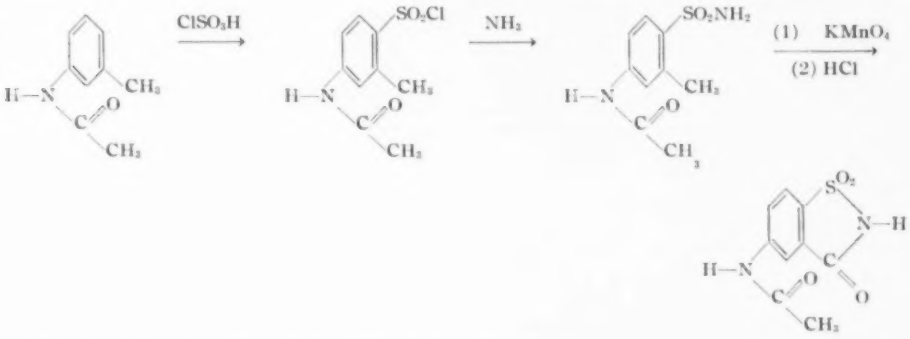
No melting point or percentage yield figures were given for the reported nitrosaccharin, but because the compound was more soluble than 6-nitrosaccharin Stubbs believed that he had synthesized a new nitrosaccharin. The 5-position was chosen because Taverne, in 1906, had nitrated *o*-sulfobenzoic acid and had reported obtaining 5-nitro-*o*-sulfobenzoic acid (4). The physical properties of the barium salt of Taverne's acid resembled those of the barium nitro-*o*-sulfobenzoate prepared by Stubbs.

On the basis of the above similarities Stubbs reported his nitrosaccharin as being 5-nitrosaccharin. Suter, who has throughly reviewed the synthesis, reactions, and derivatives of saccharin does not include this compound (5). Bambas (6)

* Received August 28, 1959, from the School of Pharmacy, University of Southern California, Los Angeles.
A portion of this work was completed at the College of Pharmacy, University of Minnesota, Minn., with the help of Dr. T. O. Soine.

in his review of saccharin chemistry does list the 5-nitrosaccharin of Stubbs.

Backeberg and Marais in 1943 reported the preparation of 5-acetamidosaccharin (7) by the following plan:



Their original objective had been the synthesis of 5-aminosaccharin, because it contains the structural elements of sulfanilamide. The authors reported that the attempted deacetylation of the 5-acetamido compound resulted in a syrup from which a crystalline product could not be obtained.

Hamor (8) has reported an attempt to prepare 5-nitrosaccharin by following essentially the procedure of Stubbs. Ammonium hydrogen *o*-sulfobenzoate was converted to the barium salt with barium hydroxide and then by the action of sulfuric acid, *o*-sulfobenzoic acid was obtained. The nitration product of this acid was obtained as the crystalline barium salt. A dilute aqueous solution of the barium salt was passed through a column of cation exchange resin and gave a soft tan-colored solid, melting in the range of 90–125°. A mixture of nitration products was evidently present. It was thought that by proceeding directly to the mixed nitrosaccharins, compounds might be obtained which would differ enough in their solubilities to be readily separated. The diacid chloride of the mixed nitro-*o*-sulfobenzoic acids was prepared by heating barium nitro-*o*-sulfobenzoate with phosphorus pentachloride. The diacid chloride was not isolated as such but was extracted by ether. The mixed nitrosaccharins were obtained as the precipitated ammonium salts by passing a stream of dry ammonia gas into the dried ethereal solution of the diacid chloride.

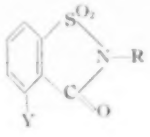
Conversion of the ammonium nitrosaccharins to the free nitrosaccharin gave a mixture of nitrosaccharins melting in the range of 196–210°. From this mixture it was possible to isolate two compounds by the difference in solubilities of their sodium salts: (*a*) A light green-colored compound possessing a slightly sweet taste and melting at 227–228° was obtained in 10% yield (for two steps), and (*b*) light yellow compound having a bitter taste and melting at 206–206.5° was obtained in a 3% yield. The sodium salt of this compound was more water-soluble than the salt of the preceding compound.

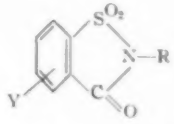
The melting point of 206–206.5° for the second compound was close to that of 6-nitrosaccharin; the compound likewise resembled it in taste and color. A mixed melting point of the two compounds showed no depression. This indicates that one position nitrated in the *o*-sulfobenzoic acid had been the 4-position, which is *para* to the carboxy group and *meta* to the sulfo group.

An investigation of the other nitrosaccharin to determine, if possible, its structure was then begun. Its melting point was close to that of saccharin (228°), but a mixed melting point of the two exhibited a depression of 40°. If the nitration of *o*-sulfobenzoic acid in this investigation were analogous to that reported by Taverne (4), this unidentified nitrosaccharin should be 5-nitrosaccharin. Backeberg and Marais (7) had reported the synthesis of 5-acetamidosaccharin (m. p. 299°) by a different route. For purposes of comparison the unidentified nitrosaccharin [hereafter referred to as (?)-nitrosaccharin] was reduced to (?)-aminosaccharin and acetylated to yield (?)-acetamidosaccharin [m. p. 332–333° (decompn.)]. A mixture of the two acetamidosaccharins showed a depressed melting point in the range of 285–290° (decompn.).

Because the unidentified acetamidosaccharin differs from that of Backeberg and Marais, the possibility that the nitro group is in the 5-position seems to be excluded. Because the 6-nitrosaccharin is well established, this leaves only the 4- and 7-positions of saccharin open for consideration. The 2-ethyl- and 2-*n*-propyl-(?)-nitrosaccharins were prepared for reference compounds by use of previously published procedures (1).

TABLE I.—PROPERTIES OF 4-NITROSACCHARIN AND THE UNIDENTIFIED NITROSACCHARIN SYNTHESIZED BY THE METHOD OF STUBBS, AND DERIVATIVES

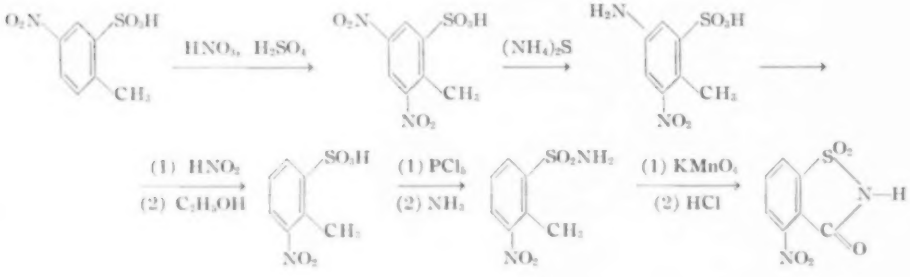




R	Y	M. P.[a], °C.	Analyses[b] — Carbon, %: Calcd.	Found	Hydrogen, %: Calcd.	Found	M. P., °C.	Analyses[c] — Carbon, %: Found	Hydrogen, %: Found
H	NO_2	236–238	36.84	36.55	1.77	1.92	241–242, mixed melt with 4-nitrosaccharin, no depression	36.88	1.80
H	NH_2	246–247	42.42	42.55	3.12	3.18	248–249, mixed melt, no depression	42.55	3.24
H		323–325[d] (decompn.)	45.00	44.96	3.36	3.38	332–333[d] decompn. mixed melt, no depression	45.06	3.65
C_2H_5	NO_2	184.5–185	42.19	42.46	3.15	3.42	184.5–185, mixed melt, no depression	42.67	3.56
C_3H_7	NO_2	138–138.5	44.44	44.55	3.73	4.02	147–149, mixed melt, no depression	44.77	4.14

[a] Melting points were taken with a Fisher-Johns melting point apparatus and are uncorrected.
[b] Analyses were performed by Elek Micro Analytical Laboratories, Los Angeles, Calif.
[c] Analyses were performed by Organic Microanalysis Laboratory, School of Chemistry, University of Minnesota, Minneapolis.
[d] Melting point done by capillary tube method.

4-Nitrosaccharin was then prepared by oxidation of 2-nitrotoluene-6-sulfonamide, which had been synthesized by the method of Hirwe and Jambhekar (9), according to the following series of reactions:



Reduction of 4-nitrosaccharin, followed by acetylation gave 4-acetamidosaccharin [m.p. 323–325° (decompn.)]. A mixture of this compound with the 5-acetamidosaccharin of Backeberg and Marais[1] melted at 282° (decompn.).

[1] The author is indebted to J. L. C. Marais, University of Witwatersrand, Johannesburg, South Africa, for supplying the sample of 5-acetamidosaccharin.

The 2-ethyl- and 2-*n*-propyl-4-nitrosaccharins were also prepared. The properties of 4-nitrosaccharin, (?)-nitrosaccharin, and their derivatives are shown for comparison in Table I. Mixed melting points of 4-nitrosaccharin and 4-nitrosaccharin derivatives with the unidentified nitrosaccharin and its derivatives showed no depression. In addition, infrared absorption spectra of the two nitrosaccharins were identical.

The above data indicate that the unidentified nitrosaccharin obtained by the method of Stubbs is identical with 4-nitrosaccharin as prepared by

the oxidation of 2-nitrotoluene-6-sulfonamide. Therefore, attempts to prepare 5-nitrosaccharin following the method of Stubbs have given instead a mixture of 4-nitrosaccharin and 6-nitrosaccharin.

It may be of interest to note that these new nitrosaccharin derivatives were tasteless, with the exception of 4-aminosaccharin which had a slightly sweet taste.

EXPERIMENTAL

4-Nitrosaccharin.[2]—This compound was prepared by alkaline potassium permanganate oxidation of 2-nitrotoluene-6-sulfonamide by the method used by Noyes (2) to synthesize 6-nitrosaccharin. The method of Hirwe and Jambhekar was used to synthesize 2-nitrotoluene-6-sulfonamide, m. p. 162°, (reported m. p. 165°) (9). Yields of approximately 30% of 4-nitrosaccharin, recrystallized from water and melting at 236–238°, were obtained. Approximately 25% of unreacted starting sulfonamide was recovered and used in succeeding oxidations.

4-Aminosaccharin.—4-Nitrosaccharin (2.0 Gm.) in 50 ml. of absolute ethanol was reduced with hydrogen at room temperature at about three atmospheres pressure with 0.7 Gm. of 5% palladium-on-charcoal catalyst. Evaporation of solvent from the filtrate and recrystallization from ethanol of the solid formed, gave 1.4 Gm. of yellow crystals melting at 246–247°. Solutions of 4-aminosaccharin exhibited a beautiful blue-purple fluorescence.

4-Acetamidosaccharin.—4-Aminosaccharin (0.8 Gm.) was dissolved in 15 ml. pyridine with aid of mild heat, cooled in an ice bath, and 5 ml. of acetic anhydride added slowly with cooling. A cream-colored solid formed immediately. The mixture after standing forty-five minutes at room temperature, was filtered and the precipitate washed with water. A crystalline solid [0.7 Gm., 323–325° (decompn.) m. p.] was obtained.

[2] Chemical Abstract nomenclature. 4-nitro-1,2-benziso-thiazolin-3-one-1,1-dioxide.

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(2) Noyes, W. A., *Am. Chem. J.*, **8**, 167(1886).
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(6) Bambas, L. L., "The Chemistry of Heterocyclic Compounds," vol. 4, Interscience Publishers, Inc., New York, N. Y., 1952, p. 350.
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(8) Hamor, G. H., Ph.D. Thesis, University of Minnesota, 1952, p. 36ff.
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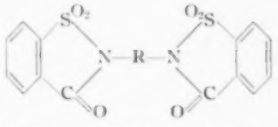
Saccharin Derivatives III*

A Note on the Synthesis of Bissaccharins

By GLENN H. HAMOR and JACK M. BALIKIAN

THE N-ALKYL and N-dialkylaminoalkyl derivatives of saccharin have a low degree of local anesthetic activity (1). The purpose of this work was the preparation of some bissaccharins for testing of various types of biological activity, including local anesthetic properties. Such testing should give information concerning the pharmacological effects of introduction of a second saccharin moiety.

The structural formulas of the seven bissaccharins prepared are illustrated.



R: CH_2, $(CH_2)_2$, $(CH_2)_3$, $(CH_2)_4$,
Compd. No: 1 2 3 4

$(CH_2)_5$, $(CH_2)_6$, $CH_2CH_2\underset{\displaystyle CH_3}{\overset{\displaystyle |}{N}}CH_2CH_2$
5 6 7

I

The saccharin derivatives were prepared according to the procedure used by Merritt, Levey, and Cutter to synthesize 2-alkylsaccharins (2). This involved refluxing sodium saccharin with an alkyl dihalide or methylbis(β-chloroethyl)amine hydrochloride in either diethylene glycol monoethyl ether-water or diethylene glycol monobutyl ether-water mixtures. After completion of this work the previous work of Reid, Rice, and Grogan describing the synthesis of all compounds except No. 7 (I), using dimethylformamide as the reaction medium, was seen (3). Therefore, this paper

* Received September 15, 1959, from the School of Pharmacy, University of Southern California, Los Angeles.

will describe only the results of the pharmacological evaluation and the synthesis of the one new bissaccharin.

Several of the compounds have undergone preliminary pharmacological evaluation. The extreme insolubility in water of these compounds has prevented testing for local anesthetic properties. Compound No. 1 produced only an insignificant degree of increased motor activity in mice after oral doses of 1,000 to 2,000 mg./Kg. Compound No. 7 was essentially devoid of biological activity in mice following oral doses as high as 2,000 mg./Kg.[1] In addition, Compounds 3 and 7 have also been screened for anticancer properties. Compound 3 gave no significant reduction in animal tumor weight in Sarcoma 180 and in Carcinoma 755. Compound 7 gave no significant reduction in animal tumor weight in Sarcoma 180.[2]

[1] The authors are indebted to Smith Kline and French Laboratories, Philadelphia, Pa., for pharmacological testing.

[2] Anticancer screening was performed through the services of the Cancer Chemotherapy National Service Center, National Institutes of Health, Public Health Service, Bethesda, Md.

EXPERIMENTAL

2,2′ - (Methyliminodiethylene)bis(1,2 - benzisothiazolin-3-one)-1,1,1′,1′-tetroxide.—To 5.0 Gm. of sodium saccharin in a mixture of 5 ml. of distilled water and 15 ml. of diethylene glycol monoethyl ether was added 0.5 Gm. of methylbis(β-chloroethyl)amine hydrochloride. After refluxing for three hours, the hot mixture was poured slowly into about 100 ml. of ice water. A precipitate was formed by making the solution alkaline to excess with sodium carbonate. Recrystallization from 95% ethanol gave 0.2 Gm. (17%) of white needle crystals melting at 156–158°.[3]

Anal.[4]—Calcd. for $C_{18}H_{19}N_3O_6S_2$: C, 50.88; H, 4.27. Found: C, 51.21; H, 4.44.

REFERENCES

(1) Hamor, G. H., and Soine, T. O., THIS JOURNAL, **43**, 120(1954).

(2) Merritt, L. L., Levey, S., and Cutter, H. B., *J. Am. Chem. Soc.*, **61**, 15(1939).

(3) Reid, E. E., Rice, L. M., and Grogan, C. H., *ibid.*, **77**, 5628(1955).

[3] Melting point was taken with Fisher-Johns melting point apparatus and is uncorrected.

[4] Analyses were performed by Elek Micro Analytical Laboratories, Los Angeles, Calif.

The Physics of Tablet Compression XII*

Extrusion and Flow Studies on Tablet Ingredients

By R. SALISBURY† and T. HIGUCHI

A method for determining the force required to extrude materials through a hole in a tablet die by means of a high force hydraulic press is presented. Extrusion forces are given for various simple inorganic electrolytes and common tablet lubricants. Extrusion of organic tablet constituents was impossible with two exceptions. Either no extrusion could be obtained at the maximum force of the press, or implosion occurred. Mixtures of sodium chloride and potassium chloride, and sodium chloride and magnesium stearate were extruded and semiempirical equations expressing behavior were evolved. Resistance to extrusion seems to correlate with the hardness of the materials under test.

DURING THE FORMATION of compressed tablets under force within a die cavity, some degree of lateral flow appears to take place. Although the exact significance of the role played by such rheological behaviors, as far as the production of tablets is concerned, is presently unknown, it would appear that more information concerning flow under high pressure of granulations and other dry forms is desirable. From the standpoint of tablet technology, it is evident, for example, that the ease with which tablets conform to the shape of the die cavity is directly dependent on the relative flowability of the material contained when under pressure.

In the present investigation, an attempt has been made to evaluate the relative flowability of compressed dry substances. As an approach in this direction the relative pressure necessary to induce extrusion of dry materials contained in a die casting through a small hole drilled normal to the direction of compression has been determined. Certain materials extruded rather smoothly when certain critical pressures were reached; others tend to expel rather violently.

* Received August 21, 1959, from the School of Pharmacy, University of Wisconsin, Madison.

† Present address: College of Pharmacy, The Ohio State University, Columbus.

This investigation was supported in part by the American Chicle Co.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

EXPERIMENTAL

Apparatus.—The extrusion studies were carried out utilizing the high force hydraulic press previously described (1). This press acted on an upper punch so that it moved into a fixed die which contained a fixed lower punch. A flat face $^{5}/_{16}$ inch punch and die set was used and, unless otherwise specified, a $^{1}/_{16}$ inch hole was drilled in the side of the annealed die immediately above the upper edge of the fixed lower punch. The die was positioned in the assembly of the press in such a manner that the extrusion opening could be observed as pressure was applied to the upper punch.

Procedure.—A known weight of material was introduced into the die, and pressure applied to the upper punch so that it entered the die and compressed the experimental material. Pressure was increased slowly and regularly until the material was extruded. At the instant that the material appeared in the extrusion opening the pressure on the upper punch was released. A Baldwin type C load cell (20,000 pounds) was used to measure the force applied to the upper punch, the signal being recorded on a Sanborn "Twin-Viso" recorder. The pressure peak on the oscillograph record was taken as the extrusion force for that weight of the substance. After each extrusion run the die was removed from the assembly and the contents were removed by driving out the compacted mass using another $^{5}/_{16}$ inch punch and a lead hammer. Punches and die were then washed in a detergent solution (a pipe cleaner serving to wash out the extrusion opening), thoroughly rinsed with distilled water, and finally rinsed with acetone, and dried in an air blast.

Preliminary experiments showed that the rate of compression had no significant effect upon the extrusion force of the particular system studied. The temperature of the room was maintained at 23°. All inorganic salts used in this experiment were dried at 110° for twelve hours and stored in a desiccator before use.

RESULTS AND DISCUSSION

Figure 1 shows a curve of the minimum force applied to the $^{5}/_{16}$ inch punch necessary to cause extrusion plotted against the weight of ammonium chloride introduced into the die. The abrupt increase in extrusion force for the smaller weights of material introduced is thought to be due to the mechanics of the extrusion procedure. As the upper punch approaches the extrusion opening, more of its force will be transmitted to the lower punch and less to the extrusion opening. Consequently, a higher total force will be required to transmit to the extrusion opening a force sufficient for extrusion in the case of small amounts of sample. Since the outer part of the curve, representing conditions where the die was nearly full of material is at a constant force, this value was used as the extrusion force for the material undergoing compression. Reproducibility in this range of the curve was found to be $\pm 4\%$.

Extrusion of Simple Inorganic Electrolytes.—The forces required for the extrusion of some simple inorganic electrolytes were determined. The relationships between the molecular diameter (calculated from the ionic radii measurements of Pauling) and the extrusion force show an inverse linear relationship (Fig. 2). The two major deviations are ammonium bromide and ammonium chloride. All the salts tested were of the cubic type, and with the exception of ammonium bromide and chloride they were all of the sodium chloride lattice type. Ammonium bromide and chloride have a cesium chloride lattice structure at room temperature (2). This is a more stable configuration and would require a proportionately higher force for extrusion. Plots of anion radius *vs.* extrusion force (Fig. 3) and cation radius *vs.* extrusion force (Fig. 4) show the same inverse linear relationship.

The smaller the ionic radii, the stronger would be the interionic forces of attraction within the crystal and, consequently, the stronger the force necessary to cause dislocations of the planes of symmetry in the crystal which would result in solid flow.

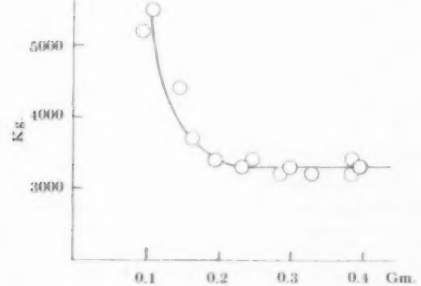


Fig. 1.—Extrusion force in Kg. *vs.* Gm. NH_4Cl.

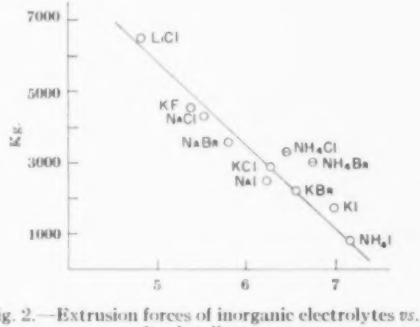


Fig. 2.—Extrusion forces of inorganic electrolytes *vs.* molecular diameter.

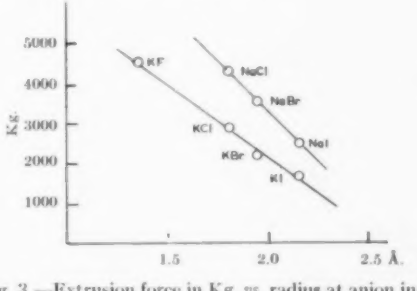


Fig. 3.—Extrusion force in Kg. *vs.* radius at anion in Å.

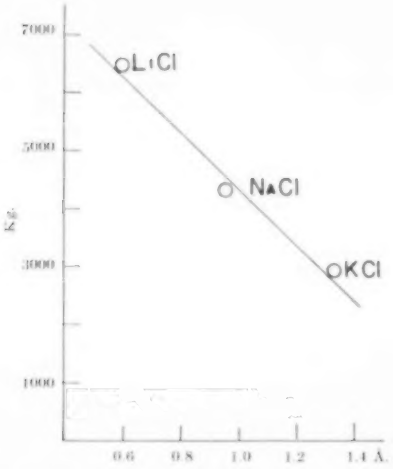


Fig. 4.—Extrusion force in Kg. *vs.* radius at cation in Å.

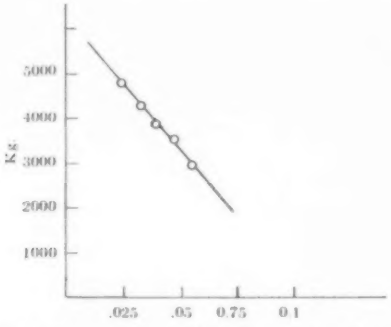


Fig. 5.—Extrusion force of NaCl *vs.* radius of extrusion opening in inches.

Extrusion Force *vs.* Radius of Extrusion Opening.—Sodium chloride was extruded through openings of varied radius. The results are shown in Fig. 5 where the extrusion force is plotted as a function of the radius of the extrusion opening in inches. The effect, as would be expected, is an inverse linear relationship, however, the effect is not a profound one.

Extrusion Force *vs.* Particle Size.—Sodium chloride in particle size ranges varying from 10–20 mesh to 140–170 mesh were extruded and the variation in extrusion forces was well within the experimental error. Thus, particle size has no significant effect on the extrusion force. Since solid flow does not occur until considerable breakdown of the original particles has occurred, it is not surprising to find no relationship between particle size and extrusion force. The major variation was due to packing of the various particle sizes in the die and the subsequent variation in weight of sample due to efficiency of packing.

Extrusion of Organic Materials.—Attempts were made to extrude a variety of organic tablet components other than lubricants (see Table I). With the exception of urea and acetophenetidin, either the materials could not be extruded at forces exceeding 7,000 Kg., or, at the points listed implosion occurred.

At first the implosion was thought to be due to binding of the material within the die, but simultaneous recording of the forces on both the upper and lower punches showed simultaneous decreases in force which could not be the case if binding was the cause of the implosion. During the compression of barbital the upper punch force was reduced by some 2,000 Kg. in 0.04 seconds. No explanation for this implosion phenomenon can be clearly demonstrated. Factors which are capable of occurring only once during a compression cycle, such as the elimination of void space, can be removed from consideration since the implosion could be repeated by recompressing the same sample. A possible explanation is the formation of a polymorph at the point of greatest resistance which is the internal opening of the extrusion hole. Figure 6 shows the lines of force formed on the lower surface of a sodium chloride sample after extrusion. The extrusion opening is shown at the bottom of the photograph and the tremendous churning effect can be seen. It is thought that at this point of highest resistance a polymorphic crystal form with a smaller molar volume might be formed resulting in a sudden drop in compressional force and that the polymorph is expelled through the extrusion opening by the resulting implosion, thus permitting the entire process to be repeated by recompression of the same sample. This theory is not borne out by the addition of lubricant to aspirin granulations (see Table I). It must be assumed that a polymorph would form at a critical pressure, and the lubricant re-

Fig. 6.—Lines of force on the lower surface of a sodium chloride sample after extrusion has occurred. The interior extrusion opening is at the bottom of the photograph and the resistance to flow is shown by the turbulence.

TABLE I.—BEHAVIOR OF ORGANIC COMPOUNDS TESTED UNDER HIGH FORCE COMPRESSION

Compound	Description	Behavior	Force Applied, Kg.
Dextrose	Unsieved powder	No extrusion at	7,600
Lactose	Unsieved powder	No extrusion at	7,600
Sucrose	20-Mesh crystals	No extrusion at	7,200
Barbital sodium	Unsieved powder	No extrusion at	7,200
Acetanilid	Unsieved powder	Implosion at	1,600
Ascorbic acid	20-Mesh crystals	Implosion at	6,800
Barbital	20-Mesh crystals	Implosion at	3,200
Caffeine citrate	Unsieved powder	Implosion at	6,400
Saccharin	Unsieved powder	Implosion at	3,300
Saccharin sodium	Unsieved powder	Implosion at	6,400
Sulfathiazole	Unsieved powder	Implosion at	4,100
Theophylline	Unsieved powder	Implosion at	3,000
Acetylsalicylic acid	Unsieved powder	Implosion at	2,650
Acetylsalicylic acid	20-Mesh crystals	Implosion at	2,650
Acetylsalicylic acid	Commercial granulation	Implosion at	2,650
Acetylsalicylic acid plus 1% magnesium stearate	Commercial granulation	Implosion at	2,200
Acetylsalicylic acid plus 2% magnesium stearate	Commercial granulation	Implosion at	1,700
Acetophenetidin[a]	Unsieved powder	Extrusion at	1,200
Urea[a]	Unsieved powder	Extrusion at	3,000

[a] One sample only.

duced the force necessary for the implosion but did not eliminate the implosion.

Extrusion of Lubricant.—A number of materials in common use as tablet lubricants were extruded and, as might be expected, the extrusion forces were quite low in comparison with other materials extruded. The results are listed in Table II and

TABLE II.—EXTRUSION FORCES FOR SOME COMMON LUBRICANTS[a]

	Kg.
Stearic acid	335
Aluminum stearate	545
Magnesium stearate	305
Calcium stearate	250
Zinc stearate	480
Talc	2,200

[a] As unsieved powder.

are noteworthy for the unexpectedly high force required for the extrusion of talc. If it is assumed that the lower the extrusion pressure, the more efficient the lubricant, the results are in excellent qualitative agreement with those obtained by measurement of the ejection force (3, 4). Other methods of evaluating tablet lubricants have dealt mainly with the effect of the lubricant on the interparticle friction or the "slip." Thus Nelson (5) found that magnesium stearate had no effect on the angle of repose of a tablet granulation while talc was effective in reducing it. Muenzel and Kaegi (6) also found talc to be better than the stearates in speeding the rate of flow of granules. The role of lubricants in tablet compression is apparently more complex than a simple reduction of the coefficient of friction between particles. Extrusion might be used as a measure of both "slip" and "antiadherence" effects of a lubricant.

Extrusion of Mixtures.—The extrusion forces for sodium chloride and potassium chloride having been demonstrated, an attempt was made to determine the variation of extrusion force with mixtures of

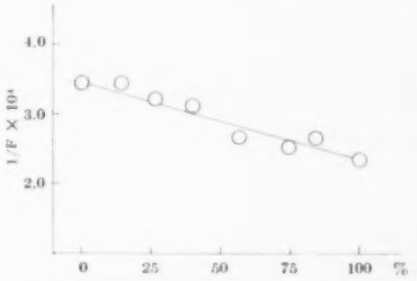


Fig. 7.—Extrusion forces of mixtures of NaCl and KCl *vs.* % NaCl.

the two salts. Accordingly, extrusion forces of mixtures of the two salts were determined. In Fig. 7, the values of the reciprocal of the extrusion forces are plotted against the percentage of sodium chloride present in the samples. The result is apparently a simple dilution phenomenon and the curve in Fig. 7 is calculated from the equation:

$$\frac{1}{F_t} = \frac{\%\ \text{NaCl}}{100\ F^\circ\ \text{NaCl}} + \frac{\%\ \text{KCl}}{100\ F^\circ\ \text{KCl}} \quad \text{(Eq. 1)}$$

where F_t is the extrusion force for the mixture and F° represents the extrusion forces of the pure components.

When the effect of adding a lubricant to sodium chloride was tested the results, when plotted as the reciprocal of the extrusion force *vs.* the percentage of magnesium stearate (Fig. 8), show a deviation from the semiempirical equation, Eq. 1. This deviation is especially marked in the low concentrations of the lubricant. The lower curves in Figs. 8 and 9 are calculated from the simple dilution formula, Eq. 1. The upper curve in each figure is derived from the equation:

$$\frac{1}{F_t} = 2\,\frac{\%\ A}{100 \cdot F^\circ_A} + \frac{\%\ B}{100 \cdot F^\circ_B} \quad \text{(Eq. 2)}$$

where F_t is again the total extrusion force, A is magnesium stearate, B is sodium chloride, and $F°$ is the extrusion force of the pure component. From Fig. 9 it can be seen that the reduction in extrusion force due to the addition of magnesium stearate is greater than is to be expected from simple dilution alone. Explicitly it is greater by a factor of two times the quotient (% lubricant/$F°$ lubricant). Thus in addition to simple dilution there is present a "lubricating" effect which is equal in intensity to the dilution factor up to about 2% magnesium stearate. Beyond this point the "lubricating factor" is reduced and the experimental points gradually return to the dilution curve, Eq. 1. This is in reasonable agreement with the maximum effective percentage of magnesium stearate usually used in tablet manufacture.

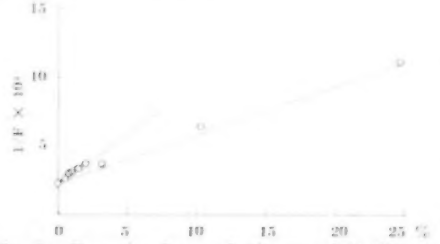


Fig. 8.—Extrusion forces of mixtures of NaCl and magnesium stearate *vs.* % magnesium stearate.

Fig. 9.—Extrusion forces of mixtures of NaCl and magnesium stearate *vs.* % magnesium stearate.

It is interesting to note that Muenzel (6) found that lubricants retarded the flow of sodium chloride crystals, while in these experiments there is a definite lowering of the extrusion force. It may be postulated that the role of the lubricant in tablet compression is more than an antiadherence factor to prevent sticking to the punches and die and an increased slip factor, which enhances the flow of the particles. It would appear from these results that the lubricant plays an additional role in decreasing the pressure necessary to obtain a desired state of compression.

Upper and lower punch force readings were recorded for several of the sodium chloride-magnesium stearate mixtures and the ratios:

$$R = \frac{\text{Lower Punch Force}}{\text{Upper Punch Force}}$$

are in agreement with literature values for the addition of lubricant (3, 4). The values obtained were somewhat low since in addition to the loss of force due to die-wall resistance there is also a loss of force through the extrusion opening.

SUMMARY AND CONCLUSIONS

1. The forces necessary for the extrusion of various inorganic electrolytes through a 1/16 inch hole in the side of a tablet die were measured.

2. For the most part, organic tablet constituents either did not extrude through a 1/16 inch opening at pressures exceeding 100 tons per square inch, or implosions occurred due to some unknown cause. Of the organic materials tested, only urea and acetophenetidin could be extruded.

3. A series of tablet lubricants were extruded and their extrusion forces are in approximate order of their efficiencies as tablet lubricants.

4. The extrusion forces for mixtures of sodium chloride and potassium chloride were found to vary according to a semiempirical formula (Eq. 1).

5. The extrusion forces for mixtures of sodium chloride and magnesium stearate were found to deviate from Eq. 1. The deviation was twice the factor representing magnesium stearate in the right side of Eq. 1 up to a lubricant concentration of 2%.

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Esters of Bicyclic Aminoalcohols II*

The Synthesis of the Hydroxyquinolizidines and Some of Their Esters as Potential Therapeutic Agents

By RAYMOND E. COUNSELL† and TAITO O. SOINE

Synthesis of esters of 1- and 3-hydroxyquinolizidine as well as of the parent aminoalcohols is described, and preliminary screening suggests that antispasmodic activity is enhanced by the *beta*-arrangement. Suitable esters of all three aminoalcohols have also been synthesized to determine the degree of local anesthetic activity associated with them. A significant degree of inhibition of spontaneous motor activity in rats was noted with the trimethoxybenzoates of 1- and 3-hydroxyquinolizidine.

A NUMBER OF SYNTHETIC DRUGS have been prepared by modification of naturally-occurring compounds. In particular, cocaine and atropine have served as prototypes for the development of numerous synthetic analogs with the hope of diminishing undesirable side effects associated with the natural products. Because these natural products are esters of complex cyclic aminoalcohols, most of the investigations to date have involved variation of the acyl portion and simplification of the aminoalcohol portion of the molecules. As a result of such investigations, a number of therapeutically useful compounds derived from rather complex acids and simple acyclic aminoalcohols have been synthesized. Nevertheless, there still appears to be room for substantial improvement in this field (1).

Sternbach and Kaiser (2) have indicated that the more complex aminoalcohols of the bicyclic type may have some merit. The aminoalcohols from which they prepared esters possessed a bridgehead nitrogen and included 3-quinuclidinol, 1-azabicyclo[3.2.1]-6-octanol, 1-azabicyclo[3.3.1]-4-nonanol, and octahydro-1-pyrrocolinol. The fluorene-9-carboxylate ester of 3-quinuclidinol was twice as active as atropine in abolishing acetylcholine-induced spasm and the diphenylacetate was equivalent to atropine. Further pharmacological data reported by Randall and co-workers (3) indicated that maximal activity was obtained in compounds wherein the nitrogen and alcoholic oxygen functions of the aminoalcohol were separated by two carbon atoms. Because of the paucity of information on esters of bicyclic aminoalcohols, Rhodes and Soine (4) were prompted to investigate the antispasmodic potentiality of esters of 2-hydroxyquinolizidine (I). This particular aminoalcohol was chosen because of its structural relationship to tropinol (II) in so far as the relative positions of the nitrogen and alcoholic functions are concerned. The only compound with significant atropine-like activity in this series, however, was the methobromide of the ester derived from xanthene-9-carboxylic acid. Nevertheless, in light of the findings of Randall and coworkers on esters of quinuclidinol (III) the preparation of esters of 1- and 3-hydroxyquinolizidine (IV and V) was indicated in the hope of potentiating the pharmacologic activity of this series. These two aminoalcohols would be isomeric with the previously examined compound but would differ in the essential aspect that each would have the desired *beta*-relationship of the nitrogen and alcoholic functions. Consequently, some esters of these two aminoalcohols have been prepared for pharmacological testing as antispasmodics or anticholinergics and, in addition, other esters of both alcohols as well as of 2-hydroxyquinolizidine have been prepared for evaluation as local anesthetics. In this latter respect, the structural relationship of piperocaine[1] (VI) [γ-(2-methylpiperidine)-propyl benzoate hydrochloride] to the benzoate of 2-hydroxyquinolizidine (VII) is worthy of note. In a study of piperidine and substituted piperidine-alkyl benzoates, it was noted by McElvain (5) that only the substituted compounds exerted any anesthetic effect on mucous membranes. The favorable pharmacological properties of piperocaine have led to its rather extensive clinical acceptance for both topical and infiltration anesthesia. Moreover, a number of compounds have been synthesized involving minor variations of the piperocaine molecule (6). A close inspection of VII reveals that it may be looked upon as a closed ring form of VI. Since an *alpha*-methyl group

* Received August 21, 1959, from the College of Pharmacy, University of Minnesota, Minneapolis.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
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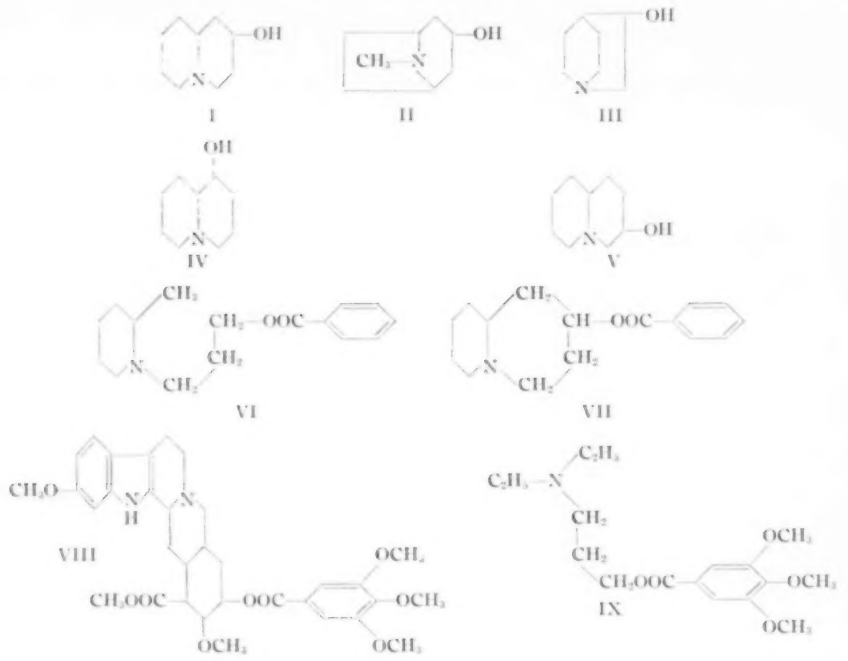
Gratitude is also expressed to the Canadian Foundation for the Advancement of Pharmacy for financial assistance.

[1] Piperocaine is marketed under the brand name of Metycaine.



on the piperidine ring increases activity, it seemed of interest to determine what effect spatial restriction, in the form of another ring, would have upon the activity of the compound. Consequently, the preparation of the benzoates of the three isomeric aminoalcohols was indicated.

Compounds containing the quinolizidine ring structure are of further interest since this nucleus is found in a number of naturally occurring alkaloids. Considering the extensive interest in reserpine (VIII), preparation of the trimethoxybenzoates of the three aminoalcohols seemed inviting. The same procedures of chemical dissection reminiscent of the tropane alkaloids, cocaine and atropine, have been applied to reserpine by Miller and Weinberg (7, 8) with the result that the γ-diethylaminopropyl ester of trimethoxybenzoate (IX) was shown to have one-third the activity of reserpine in the tests employed.

A search of the literature revealed that all three aminoalcohols had been synthesized previously. Although the 2- and 3-hydroxyquinolizidines were reported only recently (9, 10), the corresponding ketones of all three aminoalcohols have been known for some time. These were prepared by Clemo and co-workers (11–13), via the Dieckmann cyclization reaction, during the course of an extensive investigation of the lupine alkaloids. Although the Dieckmann cyclization also is utilized in this work there are enough useful modifications in procedure, resulting in enhanced yields, that these should be mentioned.

The synthesis of IV reported in the present paper is illustrated by Fig. 1. The Clemo synthesis of IV is improved considerably by the adoption of Reckhow and Tarbell's procedure (14) of alkylating ethyl piperidine-2-carboxylate with ethyl γ-bromobutyrate (easily obtained from γ-butyrolactone) instead of with the corresponding nitrile. These workers also reported an improved method for preparing ethyl piperidine-2-carboxylate which in turn has been improved in the present report. A marked improvement over the reported procedures for preparing the diester was noted when the alkylation reaction was carried out by reacting two equivalents of aminoester for each equivalent of bromoester rather than carrying out the reaction with equimolar quantites in the presence of anhydrous potassium carbonate.

The synthesis of I was carried out in essentially the same manner and in the same yields as described by Rhodes and Soine (Fig. 2) with the principal difference being that the cyclization was effected more conveniently with sodium hydride rather than metallic sodium.

Figure 3 illustrates the presently reported

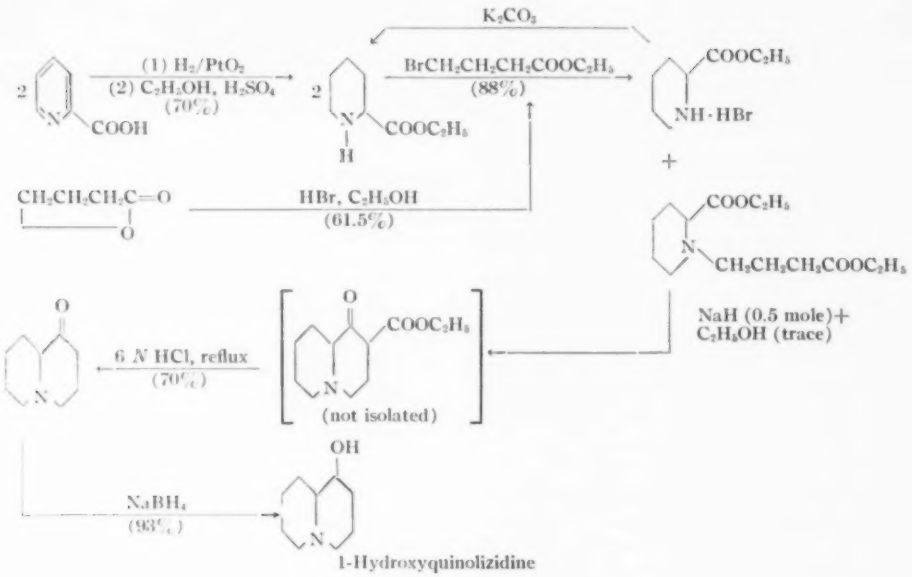


Figure 1.

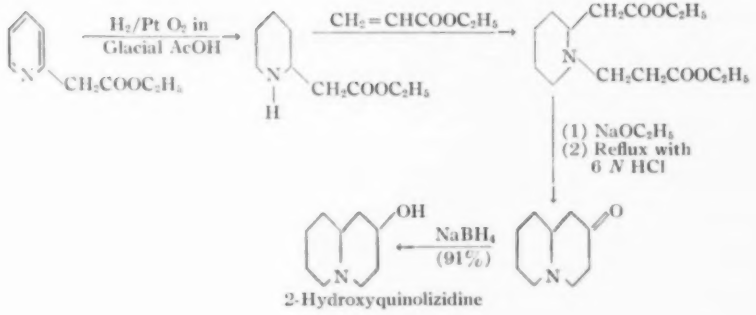


Figure 2.

synthesis of V. The method of Clemo posed a somewhat formidable problem with respect to the acquisition of sufficient diester for the cyclization reaction. These workers resorted to a somewhat lengthy procedure of condensation, hydrolysis, esterification, and hydrogenation to obtain ethyl-β-pyridyl propionate. To achieve the same goal, the procedure of Boekelheide (15) was used to obtain β-(2-pyridyl)-propionitrile by interaction of 2-vinylpyridine with acetic anhydride and potassium cyanide followed by ethanolysis of the nitrile to the desired ethyl ester. Another desirable modification of procedure was in the use of sodium hydride as a convenient condensing agent rather than either potassium or sodium metal.

In all cases the smooth reduction of the ketoquinolizidines was most easily accomplished by the use of sodium borohydride and, in analogy to other such reductions, the product is expected to be the epimer with the hydroxyl group having the equatorial conformation. Indeed, the use of platinum oxide as the catalyst in the hydrogenation of 3-ketoquinolizidine, in contrast to the quantitative hydrogenation experienced with the 2-isomer (4), resulted in hydrogenolysis with the formation of substantial amounts of quinolizidine and lowered yields of the desired compound.

The acids employed in this work were either commercially available or were readily synthesized by known methods.

Three general methods were employed for the

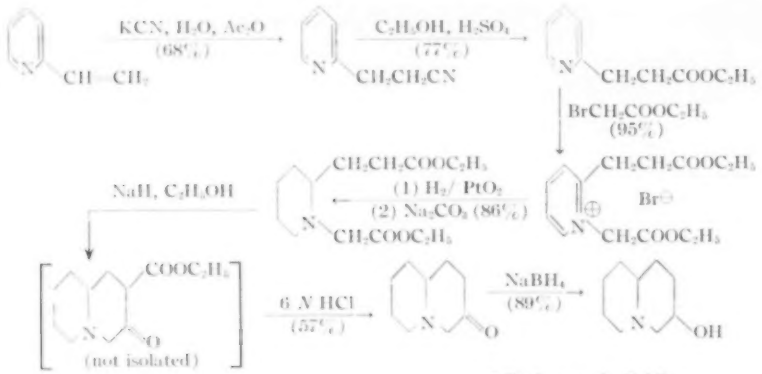


Figure 3.

preparation of the esters. In the preparation of the substituted acetic acid esters, the use of triethylamine (16) proved to be advantageous. This tertiary amine is sufficiently basic to be preferentially precipitated as the hydrochloride (method 1a). An alternate method (method 1b) was to use two moles of the aminoalcohol per mole of acid chloride. The acid chlorides were prepared in the usual manner with thionyl chloride and were employed in the crude form directly after removal of solvent and excess reagent. The acylations were performed in benzene with equimolar quantities of acid chloride, aminoalcohol, and triethylamine (or another mole of aminoalcohol). The base hydrochloride precipitated during the reaction and, on the basis of its weight, was useful for indicating the extent of reaction. When method 1 was applied to the preparation of the benzoate esters, the yields were generally poor and the products extremely difficult to purify. The Schotten-Baumann reaction also proved to be without value. These esters, however, were prepared by the ester-interchange reaction (method 2) in essentially quantitative yields and in a form easily purified. Although sodium alkoxides are the common catalysts for this reaction, sodium hydride was not only more convenient but also productive of higher yields. Contrary to Reimer and Downes (17), who stated that under the conditions of their reactions ester interchange with methyl benzoate was restricted to primary alcohols, this reaction was found to proceed well and was definitely the method of choice for the preparation of these esters. The phenylcarbamate esters were prepared in the usual manner by treating the aminoalcohols with phenylisocyanate (method 3).

The results of antispasmodic testing[2] were not as clear cut as could have been desired because the standard used (methantheline bromide) gave results indicating complete spasmolysis at a lower concentration (1:1,000,000) than partial inhibition (1:200,000). This was attributed by the pharmacologist to the vagaries of physiological test materials. However, if methantheline bromide is considered to be approximately equivalent to atropine in preventing contraction of excised ileum when stimulated by acetylcholine, the activities of some of the esters would indicate that activity of the 1- and 3-hydroxyquinolizidine esters is as great or greater than the corresponding esters of 2-hydroxyquinolizidine. The difference seems to be significant but requires more study under standardized conditions. The methobromides, as expected showed substantially more activity than the corresponding hydrochlorides. All of the compounds tested showed more activity than papaverine hydrochloride in reducing barium-induced muscle spasms. The results of the antispasmodic testing are shown in Table IV.

Local anesthetic activity of the various esters prepared is recorded in Table V using piperocaine as the standard with an arbitrary value of 1. Activity was found to be superior to that of the standard in the xanthene-9-carboxylic acid and diphenylacetic acid esters of 3-hydroxyquinolizidine, the xanthene-9-carboxylic acid ester of 1-hydroxyquinolizidine, and in the benzoate of 2-hydroxyquinolizidine. The latter showed an activity 1.72 times that of the standard, indicating that the steric restriction imposed by the ring combined with optimal interprosthetic dis-

[2] The authors are indebted to the Rowell Laboratories, Inc., Baudette, Minn., who arranged to have the testing done by the Hazleton Laboratories, Falls Church, Va.

tance could be a factor in the activity of piperocaine. Neither of the other two benzoates was significantly active. The phenylcarbamates were of uniformly low activity.

The effects of the trimethoxybenzoates of the 1- and 3-hydroxyquinolizidines on spontaneous motor activity of rats was determined by the use of activity cages. Both of the compounds depressed spontaneous motor activity in a manner qualitatively similar to chlorpromazine and reserpine but were considerably less potent. The test, however, is not specific for tranquilizing properties inasmuch as barbiturates, narcotics, antihistamines, and a host of other drugs will produce similar results. The compounds will be studied in more detail, however, in an effort to elucidate the mechanism of action. This will be reported upon by others at a later date. Table VI gives a summary of the results of testing.

EXPERIMENTAL

Synthesis of Aminoalcohols

1-Hydroxyquinolizidine.—*Ethyl Piperidine-2-carboxylate.*—This compound was prepared from picolinic acid by hydrogenation and esterification according to the procedure of Reckhow and Tarbell (14) which employs sulfuric acid as catalyst in the latter step.[3] The esterification procedure was improved somewhat by employing a Soxhlet apparatus charged with a mixture of anhydrous sodium and calcium sulfates in the extraction chamber to serve as drying agents. The yield was 68% of theory, b. p. 65–70°/2 mm., n_D^{22} 1.4554. Reported, b. p. 83–85°/7 mm. (14); 92°/12 mm. (11); n_D^{25} 1.4547 (18). Hydrochloride, m. p. 213.5–214°; reported, 202–203° (14).

Ethyl γ-Bromobutyrate.—This ester was prepared from butyrolactone in 61.5% yield by the method of Reckhow and Tarbell, b. p. 65–67°/4 mm., n_D^{20} 1.4555. Reported, b. p. 76–78°/7 mm., n_D^{25} 1.4538 (14) and 1.4539 (19).

Diethyl Piperidyl-1-γ-butyrate-2-carboxylate.—Ethyl piperidine-2-carboxylate (142.7 Gm., 0.91 mole) and ethyl γ-bromobutyrate (90 Gm., 0.46 mole) were placed in a 2-L., three-necked flask and anhydrous benzene (600 ml.) added. The flask was equipped with a mercury sealed stirrer and a condenser fitted with a drying tube, and the contents were refluxed on a steam bath for forty hours, with stirring. The mixture was allowed to cool and the precipitated salt removed by filtration. After washing the precipitate with anhydrous benzene (two 200-ml. portions), the benzene was removed from the filtrate under slightly reduced pressure. During the concentration, any additional salt that formed was removed by filtration. After removal of the benzene, the product was distilled to yield 109.3 Gm. (88%), b. p. 142–145° at 1.2 mm., n_D^{20} 1.4602. Reported, b. p. 162° at 8 mm., n_D^{20} 1.4604 (14) and 1.4588 (20). Ethyl piperidine-2-carboxylate was recovered quantitatively from the above hydrobromide salt by treatment with excess potassium carbonate solution (50% w/v), extraction with benzene, removal of solvent, and distillation under reduced pressure.

1-Ketoquinolizidine.—Dry nitrogen was bubbled through anhydrous xylene[4] (200 ml.) contained in a 1 L., three-necked flask and sodium hydride[5] (26 Gm. of a 45.8% dispersion, 0.5 mole) was then added. Commercial absolute ethanol (1 ml.) was added and the mixture heated to the reflux temperature. Diethyl piperidyl-1-γ-butyrate-2-carboxylate (54 Gm., 0.2 mole), dissolved in anhydrous xylene (200 ml.), was added dropwise with stirring over a period of two hours. Stirring at the reflux temperature was continued for an additional five hours, the mixture cooled in ice, and glacial acetic acid (34 Gm., 0.57 mole) added slowly to decompose the excess hydride. The passage of nitrogen was stopped and water (100 ml.) was added to dissolve the sodium acetate formed. The aqueous layer was separated and extracted with xylene (three 25-ml. portions) which was added to the original xylene phase. The xylene layer was then extracted three times with 6 *N* hydrochloric acid (150-ml. portions) and the combined acid extracts heated under reflux for ten hours. After cooling, the solution was neutralized and saturated with solid potassium carbonate and the excess solids collected on the filter and washed with ether. The ether washings were then used to extract the aqueous solution (five 100-ml. portions). The combined ether extracts were dried, the ether removed by distillation, and the product collected under reduced pressure. The yield of 1-ketoquinolizidine was 21.5 Gm. (70% of theory), b. p. 65–70°/0.15–0.2 mm. [reported, 95°/4 mm. (20)], $n_D^{23.8}$ 1.4912 [reported n_D^{20} 1.4935 (14)]. The picrate was prepared in ether and recrystallized from an ethyl acetate-cyclohexane mixture, m. p. 166–167° [reported, 167–168° (20)].

1-Hydroxyquinolizidine.—1-Ketoquinolizidine (21 Gm., 0.137 mole) was dissolved in water (50 ml.) and slowly added to a solution of sodium borohydride (1.5 Gm.) in water (50 ml.). The mixture was shaken and kept cool during the addition. After allowing the mixture to stand at room temperature for five hours, ammonium hydroxide solution (20 ml.) was added and the mixture allowed to stand for an additional hour. The solution was then saturated with sodium chloride and extracted with hot benzene (four 50-ml. portions). The benzene extracts were combined, dried over anhydrous sodium sulfate, and the solvent removed under slightly reduced pressure. A small portion of the white solid remaining in the flask was washed with ether and melted at 73–74° [reported, 65–68° (11)]. The remainder was distilled *in vacuo* to yield 19.7 Gm. (93% of theory) of product, b. p. 92–93°/15 mm. [reported, 127–130°/18 mm. (20)]. The picrate was prepared by precipitation from ether and recrystallization from absolute ethanol to yield yellow needles, m. p. 175.5–176.5° [reported, 174–175° (20)].

2-Hydroxyquinolizidine.—*2-Ketoquinolizidine.*—This aminoketone was prepared according to

[3] The use of absolute ethanol and dry hydrogen chloride gas in the esterification procedure resulted in a 12% yield.

[4] Redistilled and stored over calcium hydride.

[5] Obtained from Metal Hydrides Inc., 12–24 Congress St., Beverly 17, N. Y.

the method of Rhodes and Soine (4) in substantially the same yields as reported by these workers. The product corresponded in all physical properties and properties of derivatives with those reported earlier.

2-Hydroxyquinolizidine.—This compound was prepared by reduction of 2-ketoquinolizidine with sodium borohydride in the same manner as described for the preparation of 1-hydroxyquinolizidine above. Distillation of the product provided a 91% yield of a clear, colorless, viscous oil, b. p. 89–93°/0.1–0.15 mm. [reported, 132–133°/15 mm. (4)]. On cooling, the product solidified and melted at 89–90° [reported, 91–92° (4) and 92° (9)].

3-Hydroxyquinolizidine. — *β-(2-Pyridyl)-propionitrile.*—This compound was prepared by the method of Boekelheide and co-workers (15) from 2-vinylpyridine, acetic anhydride, and potassium cyanide in 68% yield as an almost colorless oil, b. p. 95–96°/2 mm., n_D^{21} 1.5180 [reported, b. p. 97–99°/2 mm., n_D^{20} 1.5175 (15); b. p. 85–87°/1 mm. (21); b. p. 93–96°/1.25–1.5 mm. (22)]. The picrate was prepared in ether and recrystallized from ethanol, m. p. 148–150° (decompn.) [reported, 140–142° (decompn.) (15, 22)].

Ethyl-β-(2-pyridyl)-propionate. — Commercial absolute ethanol (500 ml.) and β-(2-pyridyl)-propionitrile (50 Gm., 0.38 mole) were placed in a 1-L. round-bottomed flask. Concentrated sulfuric acid (50 ml.) was then slowly added to this mixture with stirring and cooling.[6] The resulting solution was placed on a steam bath, fitted with a condenser and drying tube, and refluxed for eighteen hours. At the end of this time the solution was concentrated, the viscous residue dissolved in water (100 ml.), and the resulting solution basified with ice cold aqueous 15% (w/v) sodium hydroxide solution. The mixture was then immediately extracted with ether (three 150-ml. portions) and the combined ether extracts dried over anhydrous sodium sulfate. The solvent was removed and the residue fractionated under reduced pressure. The fraction distilling at 96–100°/0.9 mm. was collected to yield 52.5 Gm. (77% of theory) of a clear, colorless liquid, n_D^{25} 1.4920. The reported b. p. is 95°/1 mm. (13). The picrate separated as an oil from ethanol and was recrystallized from ether, m. p. 83.5–84.5° [reported, m. p. 84° (13) and 83–84° (23)].

Diethyl Pyridinium-1-acetate-2-β-propionate Bromide.—Ethyl-β-(2-pyridyl)-propionate (70.0 Gm., 0.39 mole), ethyl bromoacetate (65 Gm., 0.39 mole), and anhydrous acetone (300 ml.) were placed into a 500-ml., three-necked flask and the mixture was then refluxed for eighteen hours on a steam bath. After cooling the refluxed mixture, the separated solids were removed by filtration, washed with ether, and allowed to dry. The crude product weighed 128 Gm. (95% of theory) and was used in the subsequent reaction without further purification. Recrystallization from an acetone-ethanol mixture afforded crystals, m. p., 163–164° (decompn.) [reported, m. p. 158–159° (decompn.) (24) and 159° (decompn.) (13)].

Diethyl Piperidyl-1-acetate-2-β-propionate.—Diethyl pyridinium-1-acetate-2-β-propionate bromide (20 Gm., 0.058 mole) was dissolved in water (170 ml.) and glacial acetic acid (30 ml.). The mixture was shaken with platinum oxide (0.1 Gm.) and hydrogen in a standard Parr low pressure hydrogenation apparatus. After the required amount of hydrogen had been absorbed, the mixture was filtered and the filtrate concentrated under partially reduced pressure. The resulting residue was basified with a saturated solution of sodium carbonate and immediately extracted with ether (three 125-ml. portions). The combined ether extract was dried over anhydrous sodium sulfate. After removal of the ether by distillation, the residue was fractionated under reduced pressure to yield 13.5 Gm. (86%) of a colorless liquid, b. p. 134–135°/0.9 mm., n_D^{25} 1.4634 [reported, b. p. 138–140°/1 mm. (13) and 112–113°/0.35 mm., n_D^{20} 1.4645 (24)].

3-Ketoquinolizidine.—The Dieckmann cyclization of diethyl piperidyl-1-acetate-2-β-propionate was performed in a manner almost identical to that described for the preparation of 1-ketoquinolizidine (*loc. cit.*). A 57% yield of the aminoketone was realized, b. p. 63–65°/0.25 mm., n_D^{25} 1.4910 [reported, b. p. 62–63°/0.65 mm., n_D^{20} 1.4926 (24)]. The picrate was prepared in ether and recrystallized from acetone at a low temperature, m. p. 180–181° (decompn.) [reported 180–182° (decompn.) (24)].

3-Hydroxyquinolizidine.—Reduction of 3-ketoquinolizidine with sodium borohydride in the manner described for 1-hydroxyquinolizidine (*loc. cit.*) yielded 3-hydroxyquinolizidine in 89% yield, b. p. 83–86°/0.3 mm., n_D^{25} 1.5020 [reported, b. p. 128°/14 mm. (20)]. The picrate was prepared in ether and recrystallized from ethanol, m. p. 161.5–163° [reported, m. p. 161.5–162.5° (20)].

Hydrogenation of 3-ketoquinolizidine catalytically with a platinum oxide catalyst and ferrous sulfate promoter yielded a mixture of the expected 3-hydroxyquinolizidine (61% yield) and quinolizidine, b. p. 41–45°/0.6 mm., n_D^{25} 1.4830 [reported (25), b. p. 84°/21 mm., n_D^{20} 1.4796]. The picrate was prepared, m. p. 196.5–199° [reported, m. p. 198–199° (25)]. The melting point of this picrate was not depressed by admixture with an authentic sample of quinolizidine picrate kindly supplied by Dr. V. Boekelheide.[7]

Synthesis of Esters[8]

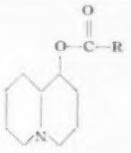
Method 1 a.—*Quinolizidin-3-yl Diphenylacetate Hydrochloride.*—The acid chloride prepared from diphenylacetic acid (3.0 Gm., 0.014 mole) and thionyl chloride (10 ml.) was dissolved in anhydrous benzene (20 ml.) and slowly added with stirring to a mixture of 3-hydroxyquinolizidine (2.0 Gm., 0.013 mole) and triethylamine (2 ml.) dissolved in anhydrous benzene (20 ml.). The reaction mixture was kept cool throughout the addition period. After allowing the reaction to proceed at room temperature for one-half hour the mixture was

[6] The use of absolute ethanol and dry hydrogen chloride gas in the ethanolysis procedure resulted in a 58% yield.

[7] Department of Chemistry, University of Rochester, Rochester, N. Y.

[8] See Tables I, II, and III for physical constants and analytical data. Because the synthesis of the esters fell into the three general methods previously discussed they will not be treated individually and only a representative synthesis of each general category is given. The example given is typical of all syntheses in that group.

TABLE I.—ESTERS OF 1-HYDROXYQUINOLIZIDINE



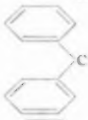
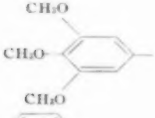
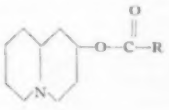
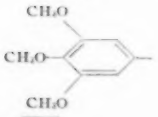
R	Method of Prepn.	Recrystallized from	M. p., °C.	Empirical Formulas	Analyses: Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
	1a	Methylene chloride, ethyl acetate, ether	161–163	$C_{23}H_{27}NO_2 \cdot HCl$	71.58	71.47	7.31	7.29
		Isopropanol, acetone, ether	234–236 (decompn.)	$C_{23}H_{27}NO_2 \cdot CH_3Br$	64.86	64.19	6.81	6.86
	1a	Methylene chloride, ethyl acetate	210–212	$C_{23}H_{23}NO_3 \cdot HCl$	69.07	68.79	6.55	6.48
		Isopropanol, acetone, ether	225–226	$C_{23}H_{23}NO_3 \cdot CH_3Br$	62.89	62.67	6.16	5.97
	1b; 2	Isopropanol, isopropyl ether	205.5–206.5	$C_{19}H_{27}NO_3 \cdot HCl$	59.14	59.24	7.31	7.30
	2	Ethanol, isopropyl ether, acetone	189–191	$C_{16}H_{21}NO_2 \cdot HCl$	64.96	65.16	7.50	7.46
	3	Ethanol, isopropyl ether	213–215	$C_{16}H_{22}N_2O_2 \cdot HCl$	61.83	61.88	7.46	7.33

TABLE II.—ESTERS OF 2-HYDROXYQUINOLIZIDINE



R	Method of Prepn.	Recrystallized from	M.p., °C.	Empirical Formulas	Analyses: Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
	2	Isopropanol, isopropyl ether	242–243 (decompn.)	$C_{19}H_{27}NO_3 \cdot HCl$	59.14	59.32	7.31	7.35
	2	Ethanol, isopropyl ether	265–266 (subl.)	$C_{16}H_{21}NO_2$	64.96	65.17	7.50	7.54
	3	Ethanol, isopropyl ether, acetone	212.5–214.5	$C_{16}H_{22}N_2O_2$	61.83	61.60	7.46	7.56

heated at 65 to 70° for two hours under a stream of dry nitrogen gas. After cooling, the precipitated triethylamine hydrochloride was removed by filtration and the residue washed with anhydrous benzene (20 ml.). The combined benzene filtrate and washings were concentrated under moderately reduced pressure and the residue dissolved in absolute ethanol. Ethereal hydrogen chloride was added to the ethanolic solution until acid to p-Hydrion paper and the solvents removed under reduced pressure. The resulting glass was hardened by triturating with anhydrous ether and recrystallized from a mixture of methylene chloride and ethyl acetate, m. p. 189–190°, yield 4.6 Gm. (92% of theory).

Quinolizidin-3-yl Diphenylacetate Methobromide.—This salt was prepared by basifying a solution of the above hydrochloride with ice cold potassium carbo-

TABLE III.—ESTERS OF 3-HYDROXYQUINOLIZIDINE

R	Method of Prepn.	Recrystallized from	M. p. °C.	Empirical Formulas	Analyses — Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
	1a	Methylene chloride, ethyl acetate	189–190	$C_{15}H_{17}NO_2 \cdot HCl$	71.58	71.69	7.31	7.22
		Isopropanol, ethanol, ether	243.5–244.5 (decompn.)	$C_{15}H_{17}NO_2 \cdot CH_3Br$	64.86	64.81	6.81	6.83
	1a	Methylene chloride, ethyl acetate	214–215.5	$C_{23}H_{29}NO_3 \cdot HCl$	69.07	69.15	6.55	6.79
		Ethanol, methanol	270–271 (decompn.)	$C_{23}H_{29}NO_3 \cdot CH_3Br$	62.89	62.62	6.16	5.99
	1a	Methylene chloride, ethyl acetate	187–188	$C_{23}H_{29}NO_2 \cdot HCl$	70.47	70.25	8.48	8.50
	1a	Methylene chloride, ethyl acetate	211–212	$C_{19}H_{27}NO_5 \cdot HCl$	59.14	59.03	7.31	7.22
	1a	Ethanol, isopropanol	255–255.5	$C_{16}H_{23}NO_4 \cdot HCl$	64.96	64.90	7.50	7.47
	3	Ethanol, isopropanol	243.5–244.5	$C_{16}H_{22}N_2O_3 \cdot HCl$	61.83	61.85	7.46	7.39

nate solution (25% w/v). The basic solution was extracted with ether and the ether extracts washed with water. After drying the extracts over anhydrous sodium sulfate, the ether was removed. The residue was dissolved in a small amount of methanol and an ethereal solution of methyl bromide added in excess. After allowing the solution to stand at room temperature in a stoppered bottle for two days, the precipitate was removed and recrystallized from a mixture of isopropanol, ethanol, and ether to give white crystals, m. p. 243.5–244.5° (decompn.).

Method 1 b.—*Quinolizidin-1-yl 3,4,5-Trimethoxybenzoate Hydrochloride.*—The acid chloride prepared from 3,4,5-trimethoxybenzoic acid (1.5 Gm., 0.0065 mole) and thionyl chloride (5 ml.) was dissolved in anhydrous benzene (20 ml.) and added to 1-hydroxyquinolizidine (2 Gm., 0.013 mole) dissolved in anhydrous benzene (20 ml.). The mixture was stirred and heated at 70° for eight hours. After cooling the reaction mixture, the aminoalcohol hydrochloride (1.3 Gm.) was removed by filtration and washed with anhydrous benzene. The benzene was removed from the filtrate under partially reduced pressure and the residue dissolved in a mixture of benzene and ether. The solution was washed with water and dried over anhydrous potassium carbonate. Ethereal hydrogen chloride was then added to the dried solution and the precipitate recrystallized from a mixture of isopropanol and isopropyl ether. This yielded 1.3 Gm. of light brown crystals, m. p. 206–209°. A second crop of crystals that precipitated from the acidulated benzene-ether mixture proved to be 3,4,5-trimethoxybenzoic anhydride, m. p. 160–162° [reported, 160–161° (26)].

Method 2.—*Quinolizidin-2-yl Benzoate Hydrochloride.*—In a 200-ml. three-necked flask equipped with an efficient stirrer, reflux condenser, Dean-Stark water separator, and a drying tube were placed Skellysolve C (40 ml.), 2-hydroxyquinolizidine (2 Gm.), and methyl benzoate (1.8 Gm.). The mixture was stirred and heated to reflux and a catalytic amount of sodium hydride paste was added. The reaction mixture was then refluxed for fifteen hours and allowed to cool. Ether (30 ml.) was added, followed by water (20 ml.), and the mixture stirred. The contents were then transferred to a separatory funnel and the aqueous phase removed and discarded. The ether-Skellysolve C solution was washed with water (four 20-ml. portions) and dried over anhydrous potassium carbonate. The solution was filtered and ethereal hydrogen chloride added to the filtrate. The product was collected on a Büchner funnel and washed with isopropyl ether. After drying, the product weighed 3.2 Gm. (84% of theory) and was readily recrystallized from isopropanol or a mixture

TABLE IV.—ANTISPASMODIC TESTING[a]

Compound	Acetylcholine Part.	Acetylcholine Comp.	Histamine Part.	Histamine Comp.	Barium Chloride Part.	Barium Chloride Comp.	Serotonin Part.	Serotonin Comp.
Quinolizidin-1-yl diphenyl acetate hydrochloride	100	500	25	500	250	500	25	500
Quinolizidin-1-yl diphenyl acetate methobromide	25	100	100	...	500	...	100	...
Quinolizidin-1-yl xanthene-9-carboxylate hydrochloride	25	500	100	500	500	...	25	500
Quinolizidin-1-yl xanthene-9-carboxylate methobromide	...	5	500	...	100	...	500	...
Quinolizidin-3-yl diphenyl acetate hydrochloride	250	...	250	...	250	...	...	25
Quinolizidin-3-yl diphenyl acetate methobromide	1	10	25	...	250	...	25	...
Quinolizidin-3-yl xanthene-9-carboxylate hydrochloride	25	150	100	...	250	...	...	25
Quinolizidin-3-yl xanthene-9-carboxylate methobromide	5	25	...	...	...	...	25	...
Quinolizidin-3-yl phenylcyclohexylacetate hydrochloride	500	1000	500	...	250	...	25	1000
Methantheline bromide	5	1	...	...	...	...	...	...
Papaverine hydrochloride	...	...	...	...	1000	...	...	...

[a] See text for explanation of values.

TABLE V.—LOCAL ANESTHETIC ACTIVITY

Compounds (as Hydrochlorides)	Anesthetic Ratio[a]
Quinolizidin-1-yl diphenyl acetate	1.01
Quinolizidin-1-yl xanthene-9-carboxylate	2.42
Quinolizidin-1-yl 3,4,5-trimethoxybenzoate	0.12
Quinolizidin-1-yl benzoate	0.54
Quinolizidin-1-yl phenylcarbamate	0.86
Quinolizidin-2-yl 3,4,5-trimethoxybenzoate	0.21
Quinolizidin-2-yl benzoate	1.72
Quinolizidin-2-yl phenylcarbamate	0.40
Quinolizidin-3-yl diphenylacetate	2.38
Quinolizidin-3-yl xanthene-9-carboxylate	3.43
Quinolizidin-3-yl 3,4,5-trimethoxybenzoate	0.10
Quinolizidin-3-yl benzoate	0.36
Quinolizidin-3-yl phenylcarbamate	0.32
Quinolizidin-3-yl phenylcyclohexylacetate	0.69
Piperocaine (*dl*-(2-methylpiperidino)-propyl benzoate)	1.00

[a] See text for explanation of values.

of ethanol and isopropyl ether, m. p. 265–266° (subl.).

Method 3.—*Quinolizidin-3-yl Phenylcarbamate Hydrochloride.*—3-Hydroxyquinolizidine (1.1 Gm.) and phenylisocyanate (1 ml.) were placed in an Erlenmeyer flask, protected with a drying tube, and heated on a steam bath for five minutes. The solid which formed upon cooling was washed with anhydrous ether and dissolved in hot ethanol. The solution was allowed to cool and ethereal hydrogen chloride added. The precipitate was recrystallized from absolute ethanol to yield 1.4 Gm. (70% of theory) of product, m. p. 243.5–244.5°. A mixture of isopropanol and ethanol also provided a suitable recrystallizing medium.

Pharmacological Testing

Antispasmodic.—Aqueous solutions of all materials were employed for this testing. The method consisted of initially standardizing the submaximal contractions of isolated guinea pig ileum to acetylcholine chloride (1 γ/100 ml.), histamine phosphate (5 γ/100 ml.), barium chloride (2 mg./ml.), and serotonin creatinine sulfate (10 γ/100 ml.). The test material was then introduced into the bath, and two minutes later the ileum was again challenged with the various spasmogens. The numbers in the table represent the γ/100 ml. of test bath for each substance tested necessary to prevent spasm when introduced into the bath two minutes before adding the spasmogen. The values under "Part." represent partial inhibition and "Comp." indicates complete inhibition. The precise value was not determined but lies somewhere in between these two values. The results are recorded in Table IV.

Local Anesthetic.—All of the compounds tested were rated against piperocaine. This compound was arbitrarily given a value of 1. The procedure used was to inject the test materials in various dilutions intradermally into the clipped backs of guinea pigs and then to determine by pin pricks the completeness of anesthesia over a period of thirty minutes. By a suitable system of scoring, a threshold anesthetic dose (dilution) of a compound producing an average of five anesthetic responses (TAD_5) was determined from a graph plotting dilution on a log scale against the average number of anesthetic responses (failure to twitch or phonate) for thirty minutes. The TAD_5 ratio was compared to that of the standard and furnished the basis for comparison of anesthetic potency. Table V summarizes the results.

TABLE VI.—SPONTANEOUS MOTOR ACTIVITY INHIBITION[a]

Compound	Dose, mg./Kg.	Number of Rats	Mean % of Initial Activity	Standard Deviation
Quinolizidin-1-yl 3,4,5-trimethoxybenzoate hydrochloride	10	6	46.8	11.0
Quinolizidin-3-yl 3,4,5-trimethoxybenzoate hydrochloride	50	6	28.3	15.9
Reserpine	10	6	2.1	2.3
Chlorpromazine	1	6	34.6	10.3

[a] See text for further explanation.

Spontaneous Motor Activity.—The tests were conducted on female albino rats in activity cages known as Actophotometers (Metro Industries). The rats, six to a group, were placed individually in the activity cages and a two-hour count was made to establish control values for spontaneous motor activity. The test compounds, dissolved in normal saline, were administered intraperitoneally and the animals replaced in the activity cages. The two-hour experimental count was begun thirty minutes after injection of the compounds. The results are recorded in Table VI.

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A Study of Certain Analgetic-Antipyretic Compounds*

By LAWRENCE C. WEAVER and BENEDICT E. ABREU

Two new compounds, α-phenyl-α-(2-piperidino-ethyl)-β-ethyl-butyric acid nitrile and 1 - phenyl - 2,3 - dimethyl - 4 - (phenyl-methyl - morpholino) - methyl - pyrazolone-(5), have been studied by various procedures. These compounds showed no analgetic activity by a thermal method in mice, weak analgetic activity as indicated by blockade of irritant-induced writhing in mice, good antipyretic activity in rats, and no antigranulomatous activity in rats.

THE DISCOVERY of therapeutically useful compounds which influence acute or chronic inflammatory conditions, and possess a strong analgetic effect as well, is one of the main goals of pharmacologic research. Analgetic and antiphlogistic activity has been reported for 1-phenyl-2, 3 - dimethyl - 4 - (phenyl - methyl - morpholino)-methyl-pyrazolone-(5)[1] by Hengen and coworkers (1), and for α-phenyl-α-(2-piperidino-ethyl)β-ethyl-butyric acid nitrile[1] by Kasparek and Pfroepffer (2). These compounds have been compared with several established compounds and the results are presented herein.

METHODS

The compound of Hengen (1) was administered in the form of its salts, e. g., as the hydrochloride (R-445-H) or as the gentisic acid salt (R-445-G); the compound of Kasparek (2) was used as the hydrochloride (R-154). These code designations will be used throughout this report. Other compounds tested are shown in the results. Male albino mice of the Swiss-Webster strain, male albino rats of the Harlan-Wistar strain, and adult mongrel dogs, unselected as to sex, were used in these studies. Animals had access to feed and water except during the period of testing. All drugs were given either as aqueous solutions or as suspensions in 1% Hercules gum[2] solution.

Analgetic Experiments.—A thermal method was used for determining analgetic activity in mice (3). All drugs for analgetic testing were administered intragastrically (i. g.) except for morphine sulfate

* Received August 21, 1959, from the Department of Pharmacology, Research Center, Pitman-Moore Co., Indianapolis, Ind.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
We are indebted to Luise Schoene and Jessa Tempke for technical assistance, and to Dr. Elva G. Shipley, Director, The Endocrine Laboratories, Madison 1, Wis., for antigranuloma testing.
[1] Supplied by Dr. H. C. Stark, Ravensberg G.M.B.A., Konstanz, Germany.
[2] Cellulose gum—CMC—120 high viscosity, Hercules Powder Co.

and normorphine hydrochloride which were given by the intraperitoneal (i. p.) route. Controls of distilled water (i. p.) and morphine were always run simultaneously with test compounds. Six animals were used at each dosage level tested. The ability of drugs to block hydrochloric acid-induced writhing in mice was also determined (4, 5). Simultaneous controls with hydrochloric acid are considered necessary for this procedure.

Anti-inflammatory Experiments.—Tests for anti-granulomatous activity were performed in 50-day-old male rats (6). The drugs were administered i. g. daily for seven days to rats previously implanted with four cotton pellets each. Autopsy was performed on the day following the last day of administration, the pellets were removed, extraneous tissue trimmed off, and pellets dried and weighed. The amount of granulation tissue formed was taken as the difference between the initial and final weight of the dried pellet. Drug effect on rat body weight as well as on granuloma weights was measured.

Antipyretic Experiments.—The effect of compounds given i. g. on normal body temperature and yeast-induced fever (7) was evaluated in rats. For temperature determinations, thermistor probes[3] were inserted rectally into male rats confined in wire mesh tubes. Two control temperature readings at thirty-minute intervals were made prior to drug injection and served as controls for each group of four animals; saline controls were run simultaneously. Temperature readings were made at hourly intervals. For experiments using fevered rats, control readings were taken prior to yeast injection (3 cc. of a 15% brewers yeast suspension in saline subcutaneously) and again prior to drug administration. The temperature of the room was maintained at 24 ± 1°.

Additional Experiments.—The ability of these compounds to block supramaximal electroshock seizures (M. E. S. test) and metrazol threshold seizures (Met. test) was determined in mice (8). Acute toxicity in mice was determined by the i. g. route. Groups of 10 mice were used at each dosage level in the determination of the LD_{50}. The LD_{50} and 95% confidence limits were determined by the method of Litchfield and Wilcoxon (9). Limited toxicity data were obtained in dogs following i. v. and i. g. administration.

RESULTS

The results of analgetic study in mice are presented in Fig. 1 and in Table I. Several compounds in nontoxic doses caused a significant ($P < 0.05$) lengthening of the reaction time of mice to a thermal stimulus. These compounds in order of potency were morphine > dihydrocodeinone > normorphine. Methylmelubrin, aminopyrine, R-445, and R-154 produced no significant increase. These last two compounds failed to alter significantly the analgetic activity of morphine sulfate.

Several compounds blocked irritant-induced writhing in mice (Table II); acetylsalicylic acid was the most potent. Both of the new compounds showed weak activity although some toxicity was observed at the more effective doses.

Phenylbutazone,[4] but not R-445 or R-154, reduced the granuloma weight in rats (Table III). In fact, the latter two compounds produced a considerable increase in granuloma weight.

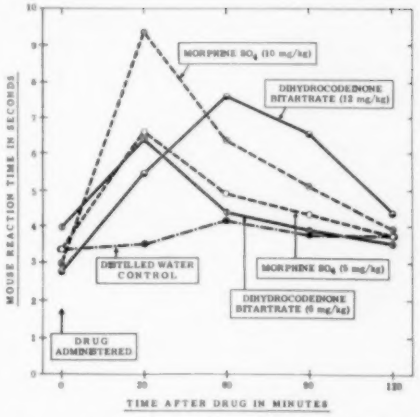

Fig. 1.—Analgetic activity of compounds in mice. All agents produced reaction times significantly greater ($P < 0.05$) than in the controls. The curves for control and morphine represent an average of five experiments.

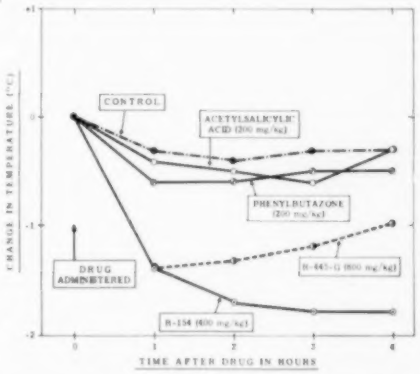

Fig. 2.—Effect of compounds on the normal body temperature of rats. Each point represents the average change in rectal temperature of eight rats.

Both test compounds produced a considerable decrease in normal body temperature of rats (Fig. 2); in additional studies at one-half these doses, only R-154 produced a significant lowering of normal body temperature. Phenylbutazone and acetylsalicylic acid were not effective at the doses tested. Both test compounds and phenylbutazone were effective in lowering the rectal temperature of rats fevered with yeast (Fig. 3).

R-154 in doses of 100 and 200 mg./Kg. i. g. showed no anticonvulsant effect. R-445-H and R-445-G were ineffective against metrazol threshold seizures; both compounds showed some protection at 200 mg./Kg. i. g. against supramaximal electroshock seizures.

[3] Yellow Springs Instrument Co.
[4] Phenylbutazone (Butazolidin) was supplied by Dr. J. Marrus, Geigy Pharmaceuticals, Yonkers, N. Y.

TABLE I.—ANALGETIC ACTIVITY IN MICE AS DETERMINED BY A THERMAL METHOD

Compound	Dose, mg./Kg.	Response Time of mice, sec. Time after Drug, min. 0	30	60	90	120
Control	..	3.76	3.83	3.37	3.49	4.10
Morphine SO_4	10	4.35	9.75	6.79	5.34	4.39
Methylmelubrin	300	3.53	4.52	4.83	4.03	4.30
Aminopyrine	100	3.66	4.32	4.29	4.33	3.70
Control	..	3.35	3.45	4.89	3.93	3.48
Morphine SO_4	10	2.63	10.15	6.47	5.29	3.80
R-154	100	3.21	4.03	3.53	4.18	3.57
R-445-H	50	4.24	3.75	4.08	4.55	4.53
Control	..	3.04	2.93	3.20	3.09	3.78
Morphine SO_4	10	3.94	8.91	6.53	4.61	3.88
Morphine SO_4	5	3.43	5.53	5.10	4.53	4.13
Normorphine HCl	40	3.35	5.25	6.33	5.19	4.43
R-154	50	3.48	3.96	4.01	4.33	3.11
R-445-G	100	3.75	3.61	4.53	3.46	3.96
Control	..	3.50	3.80	3.87	3.98	3.92
Morphine SO_4	10	3.75	10.46	6.63	5.73	4.14
Morphine SO_4	5	3.32	5.48	4.43	4.20	3.43
Morphine SO_4	5					
R-154	100	3.94	5.10	3.75	4.27	4.04
Morphine SO_4	5					
R-445-H	50	3.47	4.80	4.13	3.88	3.04
Morphine SO_4	5					
R-445-G	100	3.62	3.62	4.13	3.00	3.31

TABLE II.—BLOCKADE OF IRRITANT-INDUCED WRITHING

Drug	Dose mg./Kg.	Route	Challenging Time, min. 30	60	120	Remarks
HCl control		i. p.	$^{9}/_{10}$[a]	$^{15}/_{20}$	...	.....
Phenylbutazone	100	i. g.	..	$^{9}/_{10}$	...	.....
Phenylbutazone	200	i. g.	..	$^{5}/_{10}$	...	.....
R-445-H	50	i. g.	..	$^{7}/_{10}$	...	.....
R-445-H	100	i. g.	...	$^{4}/_{8}$	$^{4}/_{10}$	2 Mice died at 1 hour
R-445-G	50	i. g.	..	$^{10}/_{20}$	$^{2}/_{10}$	Depression at 2 hours
R-154	50	i. g.	..	$^{9}/_{10}$	..	.....
R-154	100	i. g.	$^{9}/_{10}$	$^{6}/_{10}$	$^{6}/_{10}$	Depression at 1 and 2 hours
HCl control	10	i. p.		$^{85}/_{95}$	...	.....
Acetylsalicylic acid	100	i. g.	$^{2}/_{5}$	...	...	.....
Acetylsalicylic acid	200	i. g.	$^{0}/_{5}$	...	...	.....
Acetylsalicylic acid	400	i. g.	$^{0}/_{10}$	...	...	.....
Antipyrine	200	s. c.	$^{6}/_{10}$	...	...	Marked depression
Salicylamide	200	i. g.	$^{1}/_{5}$	..	..	.....
Salicylate Na	200	s. c.	$^{8}/_{5}$	..	..	.....

[a] Number of mice writhing/number tested.

TABLE III.—DRUG EFFECT ON GRANULOMA WEIGHTS IN RATS

Treatment	Dose, mg.	Body Weights, Gm. Initial	Final	Granuloma Weights Wet, mg.	% Difference	Dry, mg.	% Difference
Controls	..	170	210	76.6	....	9.7	....
Phenylbutazone	150	176	209	70.2	− 8.3	7.1	−26.8
R-445-H	125	174	204	86.5	+12.9	12.0	+23.7
R-445-G	125	175	206	83.5	+ 9.0	10.9	+12.3
R-154	150	174	206	85.6	+11.0	11.6	+19.6

However, these latter doses were within the toxic range.

The i. g. LD_{50}s (mg./Kg.) and 95% confidence limits in mice were: R-154, 700 (636.3–770.0); R-445-H, 260 (203.1–332.8); and R-445-G, 300 (270.3–333.0). Toxic signs were those previously reported (1, 2). In one dog, R-154 at 16 mg./Kg. i. v. produced apprehension, clonic convulsions, and ataxia; duration of toxic signs was about thirty minutes. Thirty-two mg./Kg. i. v. (3 dogs) within ninety seconds produced opisthotonos and clonic convulsions lasting about five minutes, followed by occasional jerking, shaking, and ataxia for about one hour. The dogs then became sleepy and arousal was quite difficult; this condition was maintained for more than one hour. At 64 mg./Kg. i. v. (6 dogs) a tonic extensor convulsion occurred followed by the above-mentioned signs. Three dogs died

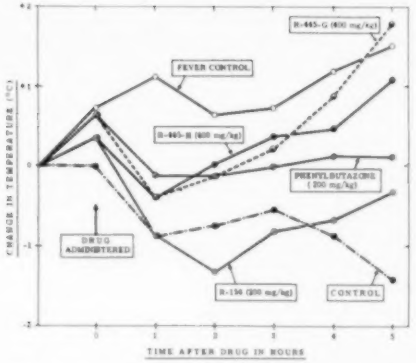

Fig. 3.—Antipyretic activity in rats. Each point represents the average change in rectal temperature of four rats.

within three minutes. At 96 mg./Kg. i. v. (1 dog) death occurred during a tonic extensor convulsion. R-445-H at 150 mg./Kg. i. g. produced emesis in dogs within ten minutes with no other toxic signs. R-445-G, 64 mg./Kg. i. g. (1 dog) produced only retching, and at 16 and 32 mg./Kg. i. p. (1 dog each) produced no toxicity. However, at 64 mg./Kg. i. p. (1 dog) emesis, clonic convulsions, opisthotonos, rigidity in front legs, involuntary jerks, and horizontally maintained ears were observed; toxic signs lasted about one hour.

DISCUSSION

Hengen and co-workers (1) reported analgetic action for R-445, aminopyrine, phenacetin, and salicylic acid amide, utilizing the analgetic test method of Wolff-Hardy. These investigators found R-445 to be two to three times as potent as aminopyrine. Similarly, Kasparek and Pfroepffer (2) found R-154 to be two to three times more analgetic than aminopyrine when evaluated by the method of electrical stimulation at the roots of the tails of mice. Furthermore, these latter investigators (2) showed that R-154 potentiated morphine analgesia. In the present studies, no significant analgetic effect was noted for aminopyrine, R-445, or R-154 by the thermal method. Furthermore, R-154 or R-445 failed to enhance the analgetic properties of morphine. It would appear that the differences might well be explained by the relative sensitivities of the various analgetic test methods used. The method used here appears satisfactory for analgetics more potent than aminopyrine or acetylsalicylic acid. We have also found acetylsalicylic acid to be quite ineffective when this thermal method for analgetic testing is used. Additional evidence to support the explanation of differences of results may be inferred by the results obtained in the writhing experiment. The well-known weaker analgetics, R-154, R-445, and phenylbutazone all showed some activity; acetylsalicylic acid was the best. Eckhardt (5) and others have observed similar effects as well as activity for the "morphine-type" analgetic. The fact that R-154 failed to alter electroshock seizure patterns of mice at a dose twice that used in the analgetic studies eliminates threshold elevation as a possible explanation of the analgetic seen.

Neither R-445 nor R-154 was effective in reducing granuloma weights in the cotton pellet implantation method in rats. On the contrary, they produced an increase in granuloma weight comparable in amount to the decrease produced by the effective compound phenylbutazone. Hengen, *et al.* (1), compared the ability of R-445 and phenylbutazone to prevent edema produced by dextran injection into the rat paw. These investigators found R-445 to be twice as effective as phenylbutazone by this method. The reason for the increase in the granuloma weights in our experiments has not been elucidated. However, similar effects have been reported for Compound S (10).

Both compounds proved to be quite effective in controlling fever as well as in lowering normal body temperature.

Considering published results and the data presented herein, it would appear that R-154 and R-445 possess (*a*) weak analgetic activity, (*b*) good antipyretic activity, and (*c*) possibly anti-inflammatory activity depending on the test used. Furthermore, the results suggest that any one test may not be sufficient for evaluating analgetic and anti-inflammatory properties.

SUMMARY

1 - Phenyl - 2,3 - dimethyl - 4 - (phenyl - methylmorpholino) - methyl - pyrazolone - (5) and α - phenyl - α - (2 - piperidino - ethyl) - β - ethyl - butyric acid nitrile have been compared with known compounds with the following results:

1. No analgetic activity using a thermal method in mice; no enhancement of morphine analgesia; analgetic activity as indicated by blockade of irritant-induced writhing in mice.

2. No antigranulomatous activity in rats.

3. Good antipyretic activity in fevered rats; lowering of body temperature in normal rats.

A possible explanation is presented for the lack of correlation of these data with previously published results.

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Paper Chromatography of Some Certified Dyes*

By F. J. BANDELIN† and J. V. TUSCHHOFF‡

Paper chromatography utilizing a single solvent system may be used to separate and identify the majority of a selected group of 20 commonly used certified dyes. Several of these dyes have similar R_f values and require other solvent systems. Color reactions for the identification and corroboration of the various dye spots are given and methods for their elution from the paper strip and quantitative determination are discussed. The method, where applicable, is rapid, requiring four to six hours for development of the chromatogram. Five- to fifty-microgram quantities of dyes can be separated and spots containing as little as 5 mcg. of dye can be readily detected with the eye.

THE USE OF certified dyes as coloring agents in pharmaceutical preparations often presents problems of separation, isolation, and identification in the analytical control laboratory. Dyes are used either alone or in mixtures to produce a variety of hues and shades, not only for esthetic appeal in products, but also for purposes of identification and differentiation in liquid, solid, and semisolid products, in solutions, syrups, ointments, creams, tablets, and capsules.

Of the 117[1] colorants on the certifiable lists of the United States, 53 are pigments and 63 are water- or solvent-soluble dyes. Since the separation of all dyes is of more theoretical than practical interest we have limited our investigation to 20 water-soluble dyes most commonly used in pharmaceutical formulations. This list of dyes corresponds reasonably well with the inventory of certified dyes for pharmaceuticals recommended by Peacock(1).

Tilden (2, 3) has reported on the paper chromatography of dyes using multiple solvents for developing the chromatograms of various dyes. We have experimented with various solvent systems attempting to develop a single solvent which might be applied as a rapid, simplified method for the separation and identification of the 20 dyes considered in this investigation. Methods of isolation and identification are also given.

EXPERIMENTAL

Materials Required.—Filter paper, Whatman No. 1 for paper chromatography, 45 cm. × 3 cm. strips.

Developing Solution.—An aqueous solution prepared by adding 2 ml. of 28% ammonium hydroxide and 2 ml. of isobutanol to sufficient distilled water to make 100 ml. Amyl alcohol C. P.

Dye Solutions.—One per cent solutions of the following certified dyes: FD&C Orange No. 1, D&C Orange No. 3, D&C Orange No. 4, FD&C Yellow No. 1, FD&C Yellow No. 5, FD&C Yellow No. 6, FD&C Green No. 1, FD&C Green No. 2, FD&C Green No. 3, D&C Green No. 5, FD&C Blue No. 1, FD&C Blue No. 2, D&C Blue No. 4, FD&C Red No. 1, FD&C Red No. 2, FD&C Red No. 3, FD&C Red No. 4, D&C Black No. 1, D&C Brown No. 1, FD&C Violet No. 1.

Procedure.—To determine the R_f values of the individual dyes under consideration, the standard dye solution is spotted on the starting line, a point 13 cm. from the lower end of the paper strip. At the midpoint on this line, a spot of dye solution, about 0.5 cm. in diameter is applied by means of a glass capillary containing the solution.

The solution is transferred to the paper by merely touching the end of the capillary and is absorbed into the paper. About 8 cm. below the starting line two slits, 1 cm. apart and parallel to the major axis, are cut in the paper. These slits are of sufficient length to form a loop in which may be inserted a piece of glass rod 3 cm. in length. Thus weighted, the strips are suspended in a large glass jar either by fastening to the under side of the cover with Scotch tape or any other appropriate method so that the strips hang perpendicularly without touching the sides of the jar or each other, and so that the weighted end is immersed to the extent of about 3 cm. into the solvent which travels vertically, by capillary action, through the paper to form ascending chromatograms. The jar is tightly closed and sealed, and after a sufficient length of time has elapsed (usually four to six hours) to permit the development of the chromatogram, the strips are removed, the solvent front marked with a pencil, and the strip air-dried. The various dye spots are noted and the R_f values calculated as follows:

$$R_f = \frac{\text{Distance traversed by the dye zone}}{\text{Distance transversed by the solvent front}}$$

APPLICATION

In pharmaceutical preparations where a mixture of dyes is suspected, these dyes may be extracted from aqueous solutions with *n*-amyl alcohol after acidifying the solution with hydrochloric acid. The following procedure may be used for the determination of the individual dyes in a dye mixture used for sugar-coated tablets.

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Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

[1] Such dyes as have been decertified or changed in certification status retain their original designation in this work.

Method for Sugar-Coated Tablets.—A number of sugar-coated tablets, usually from 5 to 20, depending upon the size, are placed in a 125-ml. Erlenmeyer flask containing 25 ml. of water. The contents are swirled with a rotary motion until all of the colored sugar coating is dissolved. The solution is then decanted from the tablets into a 50-ml. volumetric flask through a small funnel with Whatman No. 1 filter paper. The volume is made up to 50 ml. by the addition of water through the filter. The solution or a suitable aliquot is transferred to a 125-ml. separatory funnel and 2 ml. of concentrated hydrochloric acid added for every 5 ml. of solution. The solution is then extracted with two 5-ml. portions of *n*-amyl alcohol. This extracts the dyes and serves to concentrate them in a smaller volume. Should further concentration be required, the amyl alcohol may be extracted with two 2.5-ml. portions of 2% ammonium hydroxide solution, then to the combined ammonium hydroxide extracts 2 ml. of concentrated hydrochloric acid are added and the dye again extracted, this time with two 1-ml. portions of amyl alcohol.

Using the technique previously described the amyl alcohol solution of the dye(s) is spotted on the paper strip with a capillary, the spot is dried in a warm-air blast, and the chromatogram developed with aqueous ammonia-isobutanol solution and air-dried. The colored spots are noted and the R_f values calculated.

Compressed Tablets and Powders.—Dyes contained in compressed tablets or powders may be isolated by extracting the powdered tablets or the powder with 2% ammonium hydroxide, acidifying with concentrated hydrochloric acid, and continuing the extraction using *n*-amyl alcohol as given under the method for sugar-coated tablets.

Liquids.—Dyes contained in aqueous liquid products may be isolated by acidifying the liquid with concentrated hydrochloric acid, 2 ml. of acid to 5 ml. of solution, and extracting the dye with *n*-amyl alcohol as given under the method for sugar-coated tablets.

DISCUSSION

As is apparent from Table I, dyes of the same color group frequently have similar R_f values, thus making separation with the recommended solvent difficult. By allowing a longer time for the chromatogram to develop, thereby having the spots traverse a greater distance, better resolution can be obtained.

FD&C Reds No. 1, 2, and 4 have similar R_f values but can be separated satisfactorily by allowing ten to twelve hours for development of the chromatogram. This longer time produces better resolution with discreet spots which, although close together, are readily discernible and identified.

FD&C Blue No. 1 and FD&C Green No. 2 have similar R_f values. These may be separated using a solvent system composed of 80 parts of phenol and 20 parts of water and allowing to develop for twelve hours.

FD&C Yellow No. 1 and FD&C Red No. 4 may be separated using a solvent composed of 100 parts of *n*-butanol, 100 parts of 2% ammonium hydroxide, and 50 parts of ethanol. This solvent gave R_f values of 0.44 and 0.20, respectively, for these dyes.

TABLE I.—R_f VALUES OF CERTIFIED DYES BY PAPER STRIP CHROMATOGRAPHY[a]

Dye	R_f Value
Orange	
FD & C No. 1	0.264
D & C No. 3	0.771
D & C No. 4	0.168
Yellow	
FD & C No. 1	0.465
FD & C No. 5	0.662
FD & C No. 6	0.710
Green	
FD & C No. 1	0.713
FD & C No. 2	0.940
FD & C No. 3	0.959
D & C No. 5	0.615
	0.291
Blue	
FD & C No. 1[b]	0.932
FD & C No. 2	0.216
D & C No. 4[b]	0.940
Red	
FD & C No. 1	0.433
FD & C No. 2	0.421
FD & C No. 3	0.102
FD & C No. 4	0.306
Black	
D & C No. 1	0.084
Brown	
D & C No. 1	0.135
Violet	
FD & C No. 1	0.745

[a] Ammonia-isobutanol-water developer.
[b] Sodium and ammonium salts, respectively, of the same base.

There appears to be little relationship between the molecular weight and the migratory characteristics of these dyes. The R_f values are easily affected by changes in solvent and temperature and to some extent by the amount of dye used. Faster moving dyes show a greater variation in R_f values than do those with lower R_f values.

Individual dyes gave discreet spots and good separation. Although relatively good separation could be obtained with 2% aqueous ammonium hydroxide solution alone, the addition of 2% isobutanol gave better resolution and more compact spots. To obtain constant and reproducible R_f values, conditions must be standardized and very closely controlled. Reference colors should be run in the same jar at the same time, since slight deviations in conditions cause the dye spots to become capricious with resultant wide variation in R_f values. Typical R_f values obtained in a number of determinations are given in Table I. These R_f values are valid for identification only when they are obtained under conditions identical to those under which they were obtained for the reference color. Such factors as the presence of extraneous material, concentration of the dye, and the presence of similar color and R_f values, all affect R_f values. As many as eight dyes can be separated successfully on a single chromatogram and identified (see Fig. 1). Separation can usually be carried out in four to six hours. Confirmation of the identity of similar or like colors may be carried out by subjecting the moist strips of paper containing the dye spots to ammonia fumes and/or hydrochloric acid fumes to develop characteristic color reactions given in Table II.

TABLE II.—COLOR REACTION OF VARIOUS DYE SPOTS OF CHROMATOGRAMS TO FUMES OF HYDROGEN CHLORIDE AND AMMONIA

Dye	HCl	NH_3
Orange		
FD & C No. 1	Violet	Dark Red
D & C No. 3	Orange	Yellow-orange
D & C No. 4	Red	Dark orange
Yellow		
FD & C No. 1	Pale yellow	No change
FD & C No. 5	Darker yellow	No change
FD & C No. 6	Reddish	No change
Green		
FD & C No. 1	Pale Yellowish-orange	Decolorized
FD & C No. 2	Pale yellowish-orange	Decolorized
FD & C No. 3	Orange	Blue
D & C No. 5	Pale green	Pale blue
Blue		
FD & C No. 1	Yellow	No change
FD & C No. 2	Darker green	Bluish-green
FD & C No. 4	Pale yellow	No change
Red		
FD & C No. 1	Slightly paler	No change
FD & C No. 2	Slightly darker	No change
FD & C No. 3	Orange yellow	No change
D & C No. 4	Darker red	Orange-yellow
Black		
D & C No. 1	Bluish-green	Blue
Brown		
D & C No. 1	Reddish-brown	Yellowish-brown
Violet		
FD & C No. 1	Yellow	Decolorized

The most positive identification is by reference to spectrometric curves. For the latter, dyes can be eluted from the paper with 50% ethanol containing 0.5% of sodium acetate after cutting out the section of paper containing the spot, macerating it in a centrifuge tube, centrifuging, and reading the clear, supernatant liquid. Spectrometric curves plotted on solutions can be compared with standard reference curves for these dyes (4).

From 5 to 50 mcg. of the dye can be easily separated with the 5-mcg. spot producing sufficient color to be detectable by the eye.

Fig. 1.—Position of spots given by various dyes; reading from the top of the strip down.

Strip No. 1: FD&C Blue No. 1, D&C Orange No. 3, FD&C Yellow No. 5, FD&C Yellow No. 1, FD&C Red No. 4, FD&C Blue No. 2, D&C Orange No. 4, FD&C Red No. 3.

Strip No. 2: FD&C Blue No. 1, D&C Orange No. 3, FD&C Yellow No. 5, FD&C Red No. 1, FD&C Red No. 4, D&C Orange No. 4.

Strip No. 3: D&C Orange No. 3, FD&C Yellow No. 5, FD&C Yellow No. 1, D&C Orange No. 4.

Strip No. 4: FD&C Blue No. 1, FD&C Red No. 2, FD&C Red No. 3.

Strip No. 5: FD&C Red No. 1, FD&C Red No. 4, D&C Brown No. 1, D&C Black No. 1.

Strip No. 6: FD&C Violet No. 1, FD&C Red No. 2, FD&C Red No. 3.

Strip No. 7: D&C Orange No. 3, FD&C Red No. 2, FD&C Orange No. 1, D&C Orange No. 4, FD&C Red No. 3.

Strip No. 8: FD&C Green No. 2, FD&C Green No. 1.

SUMMARY

The separation and identification of 20 commonly used certified dyes can be achieved, with few exceptions, through paper chromatography using a single solvent system composed of 2 per cent aqueous ammonium hydroxide solution containing 2 per cent isobutanol. Because of discrepancies of the R_f values obtained for the dyes due to variation of conditions and techniques during development of the chromatogram, R_f values alone cannot be relied upon for positive identification of the dye. For this reason, confirming colorimetric tests and spectrometric reference curves are relied upon for definite identification.

Methods for the extraction and concentration of the dyes from certain pharmaceutical dosage forms are given along with the general method for the chromatography of the dyes.

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Adsorption of Lipid-Soluble Substances by Human Keratin*

By DALE E. WURSTER and ROBERT E. DEMPSKI

The adsorption of various lipid-soluble alcohols, esters, acids, and mixtures from *n*-heptane solutions on columns composed of powdered, excised, human callous tissue was investigated. Experimental evidence indicates that unsaturated and 2-hydroxy fatty acids were adsorbed. Infrared analysis substantiated the selective adsorption of acids from mixtures composed of human skin lipids and wool fat acids.

MANY LIPID-SOLUBLE SUBSTANCES are commonly applied to the surface of human skin in the form of pharmaceutical and cosmetic preparations. Natural human skin lipids, of course, are also in intimate contact with the keratin layer of skin. Several studies have dealt with the adsorption of various ions by protein (1–4) and the composition of human skin lipids has also been extensively investigated (5, 6). However, little information is available regarding the possible adsorption of the components of skin lipids or other lipid-soluble substances by human keratin.

This investigation was, therefore, initiated in an attempt to determine whether keratin would adsorb certain pure acids, alcohols, and esters and whether selective adsorption would occur from lipid-soluble mixtures such as skin lipids, Lantrol, and wool fat acids.

EXPERIMENTAL

Procedure.—Excised human callous tissue[1] which had not been treated with chemical agents prior to removal was used as a source of keratin. Although certain differences in composition may exist between callous tissue and the normal keratin layer of the skin, callous tissue was the only type that could be obtained in adequate amounts for this study. The keratin was dried for four days over concentrated sulfuric acid and then reduced to a powder in a wedgewood mortar. The powder was fractionated by passing it through standard 100-, 140-, and 200-mesh sieves. The skin lipids were then completely removed from the powder by continuous extraction with anhydrous reagent grade ether for forty-eight hours at room temperature. Since no heat was employed in the extraction procedure, one pound of ether was used to extract each 2.0-Gm. sample of keratin.

The density of the extracted keratin was found to be 1.19 by the pycnometer method using ether as the displacing fluid. The average particle diameter of the collected fractions was determined with a Fisher sub-sieve sizer (model 14-312) using 0.65, 0.60, and 0.55 porosities.

A known weight of powdered keratin was slurried in purified *n*-heptane and placed in an adsorption column having a 12-mm. diameter and 38-cm. length. Solutions containing various lipid-soluble acids, alcohols, esters, and mixtures in purified *n*-heptane were passed through the columns at the rate of 1 ml. per five minutes. Following this, *n*-heptane was passed through the column until only the pure solvent was obtained.

The amount of adsorbed material was found from the difference between the total amount of material placed on the column and the weight of unadsorbed material. The adsorbed material was eluted from the column with anhydrous ether and subjected to infrared analysis on a Beckman I. R.-5.

In the case where the adsorption columns contained hydrated keratin, the desiccated keratin was first exposed to an atmosphere saturated with water vapor in a closed vessel at room temperature for one to four days. Samples containing 22 to 46% water were thus obtained. Adsorption studies on the hydrated keratin were then carried out according to the previously described procedure. However, it was also necessary to run a blank in this case as a small amount of water-soluble material (0.1–0.3 mg. per 5-ml. fraction), which may have been free amino acids (7), was removed from the column.

RESULTS AND DISCUSSION

Table I shows the various substances which were tested for adsorption on columns composed of both dehydrated and hydrated keratin. As indicated, of the substances tested, only unsaturated (linoleic and linolenic) and hydroxy (2-hydroxystearic) fatty acids and a fraction of the wool fat acids and skin lipids were adsorbed. In an initial study, oleic acid appeared to adsorb weakly on keratin; however, this could not be substantiated in subsequent tests. None of the lipid-soluble esters, alcohols, or Lantrol components appeared to be adsorbed.

After elution of the adsorbed fatty acids with ether the keratin was again capable of adsorbing these acids. However, particle size measurements on this reprocessed keratin showed an increased average diameter which was probably due to aggregation of the fine powder.

Linoleic, Linolenic, and 2-Hydroxystearic Acids.—In Table II it can be observed that 2-hydroxystearic acid was more strongly bound than linolenic, and the latter was bound more strongly than linoleic. The adsorption of certain aromatic hydroxy acids

* Received August 21, 1959, from the School of Pharmacy, University of Wisconsin, Madison.

This study was supported in part by a grant from the Malmstrom Chemical Corp., Newark, N. J.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

[1] The authors are grateful to the chiropodists of Madison, Wis., especially Dr. R. D. Harmon, and to the Chicago, Ohio, and Illinois Colleges of Chiropody for the callous tissue used in this investigation.

TABLE I.—ADSORPTION OF VARIOUS LIPID-SOLUBLE SUBSTANCES ON DEHYDRATED AND HYDRATED KERATIN[a]

Test Substance	Dehydrated Keratin	Hydrated Keratin[b]
Cholesterol	−	−30
Stearyl alcohol	−	−31
Ricinoleyl alcohol	−	...
Cholesteryl palmitate	−	−26
Tristearin	−	−32
Wool fat acids	+	+30
Stearic acid	−	−36
Palmitic acid	−	−28
Oleic acid	−	−22
Linoleic acid	+	+25
Linolenic acid	+	+35
2-Hydroxystearic acid	+	+30
Lantrol	−	−46
Human skin lipids	+	+27

[a] +, adsorption; −, no adsorption.
[b] Per cent water present.

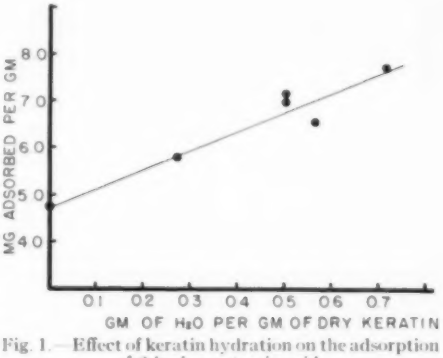

Fig. 1.—Effect of keratin hydration on the adsorption of 2-hydroxystearic acid.

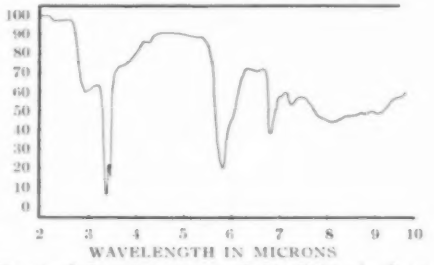

Fig. 2.—Infrared spectrum of skin lipid adsorbate.

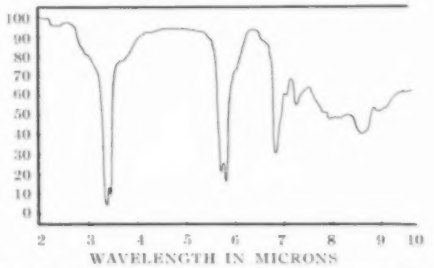

Fig. 3.—Infrared spectrum of whole skin lipids

on wool protein was previously reported by Bradley and Easty (3) but no mention of the adsorption of the above fatty acids on human keratin was found in the literature.

Hydrated Keratin.—Since human keratin normally contains water, the effect of hydration of the keratin on adsorption was also studied. In this case 2-hydroxystearic acid which was the most strongly adsorbed acid investigated was employed. Figure 1 is a plot of the mg. of acid adsorbed per Gm. of keratin (average particle diameter 4.3 μ) *versus* the amount of water per Gm. of dry keratin after hydration. From these data it appears that hydration of the keratin causes an increase in the amount of the acid adsorbed. This increase may possibly be explained on the basis of the unfolding of the protein molecule following hydration which may then expose more functional groups.

Human Skin Lipids.—The human skin lipids used in this work were obtained by the method described by Tingstad, Wurster, and Higuchi (8). According to Wheatley (5) skin lipids contain 28% free fatty acids consisting mainly of stearic, oleic, and palmitic acids and smaller amounts of linoleic, linolenic, and others.

The adsorption data for human skin lipids are given in Table II. The concentration (46.3 mg./10 ml.) used in these determinations represented the

TABLE II.—ADSORPTION DATA FOR VARIOUS FATTY ACIDS AND HUMAN SKIN LIPIDS ON DEHYDRATED KERATIN

Test Substance	Wt. of Keratin Column, Gm.	Av. Diameter of Keratin, μ	Concn. of n-Heptane Solution	mg. Adsorbed per Gm. of Keratin
Linoleic acid	8.32	5.05	48.4 mg./10 ml.	0.42
	10.29	19.5	48.4 mg./10 ml.	0.41
	11.91	22.9	48.4 mg./10 ml.	0.36
Linolenic acid	7.81	5.1	42 mg./10 ml.	0.56
	9.80	20.1	42 mg./10 ml.	0.56
	11.71	23.2	42 mg./10 ml.	0.42
2-Hydroxystearic acid	6.92	5.85	8.9 mg./15 ml.	0.88
	9.06	23.3	8.9 mg./15 ml.	0.66
	11.33	26.8	8.9 mg./15 ml.	0.52
Human skin lipids	5.94	6.1	43.3 mg./10 ml.	0.37
	7.97	23.7	43.3 mg./10 ml.	0.34
	10.61	27.0	43.3 mg./10 ml.	0.31

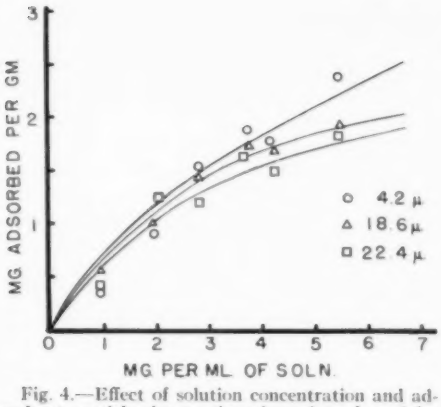

Fig. 4.—Effect of solution concentration and adsorbent particle size on the adsorption of wool fat acids.

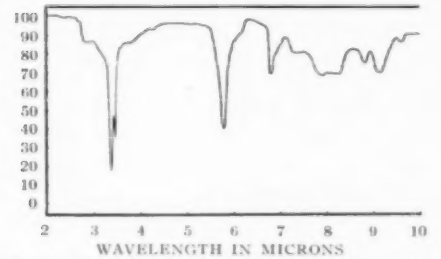

Fig. 5.—Infrared spectrum of wool fat acids adsorbate.

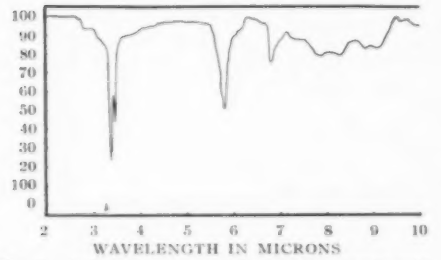

Fig. 6.—Infrared spectrum of 2-hydroxystearic acid.

n-heptane-soluble fraction from 52.9 mg. of whole skin lipids. To confirm the identity of the material adsorbed on the keratin column an infrared spectrum was obtained (Fig. 2). For comparison, a spectrum of whole skin lipids is shown in Fig. 3. The 5.7 μ peak is characteristic of esters and the one at 5.8 μ is characteristic of acids. Thus, the 5.8 μ peak indicates that the skin lipid adsorbate is composed mainly of acids.

Wool Fat Acids.—A mixture of wool fat acids was used to determine both the effect of varying the concentration of the acids in the *n*-heptane solution and the effect of the particle size of the keratin on the amount of acid adsorbed. According to Weitkamp (9), wool fat contains 32 acidic components. These were classified as normal fatty acids, 2-hydroxy acids, iso acids, and anteiso acids. Horn (10) reported that 2-hydroxy acids represent 27.8% of the wool fat acid mixture. In Table III it can be observed that the keratin adsorbed from 13.5 to 24.3% of the acid mixture placed on the column.

TABLE III.—ADSORPTION DATA FOR WOOL FAT ACIDS ON DEHYDRATED KERATIN

Concn. of *n*-Heptane Solutions, mg./5 ml.	Av. Diameter of Keratin Powder, μ	mg. Adsorbed per Gm. of Keratin	% of Total Acids Adsorbed
4.7	4.2	0.35	14.9
9.8	4.2	0.9	18.4
14.0	4.2	1.55	22.2
18.6	4.2	1.9	20.5
20.9	4.2	1.8	17.2
27.2	4.2	2.4	17.6
5.0	18.6	0.55	22.0
9.9	18.6	1.0	20.2
14.1	18.6	1.45	20.6
18.9	18.6	1.75	18.5
21.2	18.6	1.7	16.0
27.4	18.6	1.95	14.2
4.8	22.4	0.4	16.7
10.3	22.4	1.25	24.3
14.1	22.4	1.2	17.0
18.4	22.4	1.65	17.9
21.2	22.4	1.5	14.1
27.4	22.4	1.85	13.5

Figure 4 shows that the amount of material adsorbed increased as the concentration of the *n*-heptane solutions increased, and adsorption decreased as the particle size of the adsorbent increased, as would be expected. Colvin (4), working with other adsorbates and adsorbent protein materials, obtained similar plots.

In Fig. 5 it can be observed that the infrared spectrum is almost identical to the spectrum obtained with 2-hydroxystearic acid (Fig. 6). Of particular interest is the absorption in the 2.8 μ region which represents the hydroxyl group and which is present in both cases.

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Yellow Phenolphthalein II*

By MAX H. HUBACHER

Two of the by-products formed in the commercial synthesis of yellow phenolphthalein have been isolated and tentatively characterized. Degradation studies have provided subsequent compounds which have been shown to be identical with authentic samples prepared by direct synthesis. Additional evidence is presented through evaluation of data from infrared spectroscopy.

YELLOW PHENOLPHTHALEIN is a widely used laxative, manufactured by condensing phenol with phthalic anhydride. It is of yellow color, contains at least 94 per cent phenolphthalein and several other compounds that have been isolated and identified (1).

Two other compounds have also been isolated from yellow phenolphthalein, each in amounts of less than 0.1 per cent, one melting at 250° (V) and the other at 254° (X). Compound V has been briefly described previously (1). Much experimental information has been collected on the two compounds. Even though their structural formulas have not been definitely established, it is felt that the information collected so far should be recorded. Circumstances prevent a continuation of this work.

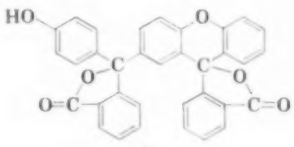
Compound V, $C_{34}H_{20}O_6$, has two lactone groups and one OH-group, and melts at 250°. On reduction of V, a dicarboxylic acid (Vd) is formed. All of this has already been reported in the first paper on yellow phenolphthalein. Now, a monobenzoyl derivative of V has also been made. When the dicarboxylic acid Vd was decarboxylated and the resulting oil distilled with zinc dust, a well-defined compound (VI), $C_{26}H_{20}O$, melting at 126°, was obtained. This colorless compound is neither soluble in dilute aqueous alkalies nor in concentrated sulfuric acid. Spectroscopic examination of VI suggests it to be a derivative of 9-phenyl- or 9-benzylxanthene. It was found to be identical with 2-benzyl-9-phenylxanthene, the synthesis of which is described.

The formation of 2-benzyl-9-phenylxanthene gives support to the formula suggested for V. The carbon skeleton of this xanthene derivative VI can also be detected in V. It is admitted that structural evidence obtained by zinc dust distillation must always be evaluated with caution, but in the phthalide field, this reduction method has been helpful in structure evaluation (2).

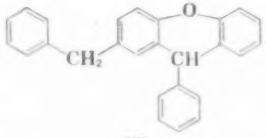
Compound X melts at 252–255°, has the probable empirical formula of $C_{34}H_{22}O_7$, and contains one mole more water than V. Neither the acetyl- nor the methylether could be obtained in crystalline form. However, the determination of active hydrogen suggests that X has three OH-groups. The new compound forms solvate crystals with various solvents. The one with two moles of acetic acid was studied especially. Compound X dissolves in dilute aqueous alkalies with the same pink color as phenolphthalein.

On KOH fusion of X, phenol, benzoic acid, and *p*-hydroxybenzoic acid were obtained. The pink color of an alkaline solution of X fades very slowly (3), and *o*-(*p*-hydroxybenzoyl)-benzoic acid could be isolated. When X was subjected to the Dakin reaction, i. e., treatment with alkaline hydrogen peroxide (3), then phthalic acid, hydroquinone, and 3-(*p*-hydroxyphenyl)-3-(*m*,*p*-dihydroxyphenyl)-phthalide (XI), also named phenolcatechol-phthalein, were obtained. The formation of XI is evidence that phenolphthalein is part of the molecule of X, and that it is substituted in *ortho*-position to one of its hydroxyl groups. When X is reduced, a dicarboxylic acid XII is formed, proof that X has two lactone groups.

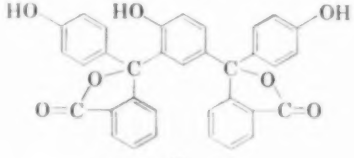
The following tentative structural formulas are given for V and X:



V



VI



X

* Received June 11, 1959, from the Research Laboratory of Ex-Lax, Inc., Brooklyn 17, N. Y.
The author wishes to acknowledge the experimental assistance of Douglas Curtin.

The formula for V suggests that this compound may be synthesized from *o*-(*p*-hydroxybenzoyl)-benzoic acid and fluoran; compound X from the same aicd and phenolphthalein. It should be remembered that the *o*-(*p*-hydroxybenzoyl)benzoic acid is an intermediate in the formation of phenolphthalein from phenol and phthalic anhydride (4).

Some 2-hydroxyanthraquinone could be isolated from yellow phenolphthalein, but only in quantities of less than 0.01 per cent. This small amount of this yellow compound is not responsible for the main yellow color of yellow phenolphthalein. The yellow compound or compounds mainly responsible for the yellow color of yellow phenolphthalein have not been isolated. They seem to be quite elusive.

EXPERIMENTAL[1]

Isolation of the compounds.—The isolation of compounds from yellow phenolphthalein was done essentially as described on page 264 of the first paper (1). The brownish, amorphous residue, which was left after no more crystals could be obtained, was dissolved in absolute ether. The yellowish-brown solution, showing greenish fluorescence, was passed through a Tswett column, 4 × 80 cm. inside dimensions, filled with Florisil (100/200 mesh, Floridin Co., Tallahassee, Florida). The first percolates contained phenolphthalein and isophenolphthalein or 3-(*o*-hydroxyphenyl)-3-(*p*-hydroxyphenyl)phthalide. Then came percolates with compound V, and finally percolates with X in them. The pink layer of the ether-washed column, on extraction with ethanol, usually yielded additional small quantities of X.

Separation on the column is not sharp and sometimes, crystal mixtures of V and X are obtained. Separation of such mixtures is easily effected since only compound X is soluble in 0.1 *N* sodium hydroxide.

Benzoylderivative of V.—By warming 0.20 Gm. of V, 0.24 ml. pyridine, and 0.15 ml. benzoylchloride, and recrystallizing the crude benzoylderivative several times from ethanol, very fine needles arranged in warts and melting at 207.1–209.8° were obtained.

Anal.—Calcd. for $C_{41}H_{24}O_7$: C, 78.34; H, 3.82; one benzoyl 16.7. Found: C, 78.56; H, 4.11; benzoyl 16.8 ± 1.9.

Compound VI.—Compound V was reduced to dicarboxylic acid Vd, and the latter decarboxylated to the oily compound, as described on page 265 of the first paper (1).

The oil obtained by decarboxylation of 1.7 Gm. of Vd, was mixed with 5 Gm. of $ZnCl_2$, 1.0 Gm. NaCl, and 3.0 Gm. of zinc dust (5) and the mixture gradually heated in an atmosphere of hydrogen to 260° A yellowish, fluorescent oil condensed on the cold part of the tube. This oil slowly crystallized. This new compound, after several crystallizations from ethanol and sublimation at 120° and 10 μ pressure, formed colorless crystals melting at 125.8–126.5° (VI). It is insoluble in both 1 *N* sodium hydroxide and concentrated sulfuric acid.

Anal.—Calcd. for $C_{26}H_{20}O$: C, 89.62; H, 5.78 mol. wt. 348. Found: C, 89.36 ± 0.26; H, 5.79 ± 0.14; mol. wt. 351; 316 (Rast).

A mixture of this compound VI with 2-benzyl-9-phenylxanthene (m. p. 126.0–126 5°) melted at 126.0–126.6°.

Compound X.—The compound crystallizes from acetic acid (1 Gm. in 18 ml.) in fine needles which give off acetic acid around 135° with effervescence, then melt at 252.2–254.4° (X). A mixture of X with phenolphthalein (m. p. 261°) melts at 225–235°. These crystals from acetic acid dried first at 25°, then to 150° in vacuum, lose 17.4 ± 0.7% in weight, and the liquid coming off is acetic acid (Calcd. for $C_{24}H_{22}O_7 \times 2\ CH_3COOH$: 18 1%).

Anal. of crystals obtained from acetic acid and dried at 25°.—Calcd. for $C_{24}H_{22}O_7 \times 2\ CH_3COOH$: C, 68.88; H, 4.53. Found: C, 69.17 ± 0.87; H, 4.67 ± 0.14.

Anal. crystals, after drying at 150° *in vacuo*.—Calcd. for $C_{24}H_{22}O_7$: C, 75.27; H, 4.06; mol. wt. 542; three H, 0.55. Found: C, 75.55 ± 0.32; H, 4.46 ± 0.12; mol. wt. 583 ± 63; active H, 0.46.

These analytical figures also check with the empirical formulas $C_{25}H_{24}O_7$ and $C_{26}H_{26}O_7$.

Compound X also can be recrystallized from acetone, forming rectangular crystals containing solvate acetone. It dissolves in 0.1 *N* sodium hydroxide with the same pink color as phenolphthalein in the same concentration.

When a solution of 0.1 Gm. of X in 0.5 ml. 5 *N* sodium hydroxide is heated in a sealed glass tube for twenty-four hours to 100°, most of X is recovered unchanged.

When 0.4 Gm. of X was added to 5 Gm. molten KOH at 230–235° and the melt stirred for five minutes, then phenol, benzoic acid, and *p*-hydroxybenzoic acid were obtained.

The red solution made up from 1.0 Gm. of X in 6.6 ml. 0.2 *N* sodium hydroxide was kept for four months in an atmosphere of oxygen at room temperature. The brownish solution was then found to contain *o*-(*p*-hydroxybenzoyl)-benzoic acid.

To a solution of 1.0 Gm. of X in 14 ml. sodium hydroxide, 2.0 ml. 30% hydrogen peroxide was added. The temperature went up slowly to a maximum of 47°, the color changed to a bluish-red, and a precipitate formed. The solution was saturated with CO_2. The water-soluble phenol consisted of 31 mg. hydroquinone. The water-insoluble brown phenol (0.33 Gm.) was acetylated and the acetylderivative recrystallized several times from ethanol; it melted at 149–151° (XI b) and the melting point was not depressed when mixed with the triacetylderivative of 3-(*p*-hydroxyphenyl)-3-(*m,p*-dihydroxyphenyl)-phthalide of m. p. 154.5–155.6°. Kin (6) gives an m. p. of 148° for this triacylderivative. XI b on hydrolysis in 1 *N* sodium hydroxide yields a bluish solution, of the same shade as that of an alkaline phenol-catechol-phthalein.

Some phthalic acid was isolated also.

X reacts with acetic anhydride, but the acetylderivative could not be made to crystallize; neither

[1] All melting points are corrected. Molecular weights were determined by the Signer method, as described by Clark, E. P., *Ind. Eng. Chem., Anal. Ed.*, **13**, 820 (1941).

could the gummy methyl-ether be obtained crystalline.

Dicarboxylic Acid XII. A mixture of 1.0 Gm. of X, 20 ml. 80% acetic acid, and 1.0 Gm. zinc (30 mesh) was refluxed. When all the zinc had dissolved, an additional 1.0 Gm. zinc was added. The solution was poured into water and the gummy precipitate became hard. It was crystallized from 20% ethanol. This dicarboxylic acid melts at 258.9–259.8° (decompn.).

Anal.—Calcd. for $C_{34}H_{26}O_7$: C, 74.72; H, 4.79; mol. wt. 546. Found: C, 74.15; H, 5.10; mol. wt. 510; neut. equiv. 280.

Synthesis of 2-Benzyl-9-phenylxanthene

***o*-(*p*-Benzylphenoxy)benzoic Acid.**—The bluish solution of 15.6 Gm. (0.1 mole) of *o*-chlorobenzoic acid (m. p. 138.5–140.0°), 18.4 Gm. (0.1 mole) of 4-hydroxydiphenylmethane (m. p. 79–80°), 0.2 Gm. CuCl in 100 ml. 2 *N* of sodium methylate was heated on an oil bath. First, the methanol distilled off. The temperature was raised gradually to 200° (oil bath) and kept for one hour at that temperature. The crude acid, after one crystallization from 41% ethanol (1 Gm. in 52 ml.) weighed 22.4–24.4 Gm (yield 73–80%) and melted at 138–139°.

By subliming this acid at 135° and 8 μ pressure, it is obtained as white crystals, melting at 141.8–142.2°. Its solution in concentrated sulfuric acid is colorless but becomes bluish fluorescent on heating.

Anal.—Calcd. for $C_{20}H_{16}O_3$: C, 78.94; H, 5.26; mol. wt. 304. Found: C, 79.23; H, 5.41; mol. wt. 291; neut. equiv. 301.

The *p*-toluidide, purified by crystallizations from ethanol, melted at 119.2–121.3°.

Anal.—Calcd. for $C_{27}H_{23}N_2O$: C, 82.41; H, 5.89; N, 3.56. Found: C, 82.71; H, 5.76; N, 3.38.

The 2-naphthylamide of this acid, crystallized from ethanol, melted at 100.1–101.3°.

Anal.—Calcd. for $C_{30}H_{23}NO_2$: C, 83.89; H, 5.39; N, 3.26. Found: C, 83.81; H, 5.14; N, 3.42.

2-Benzyl-9-xanthenone.—A solution of 30.0 Gm. of *o*-(*p*-benzylphenoxy)benzoic acid, 80 ml. of acetyl chloride, and 0.5 ml. of concentrated sulfuric acid (7) was heated to a final temperature of 95°, distilling off HCl and excess acetyl chloride. On adding water to the cooled solution, the xanthenone crystallized out. It was dissolved in 800 ml. ethanol at reflux temperature and then half of the solvent was distilled off. The yield was 26.0 Gm. (92%), m. p. 132–134°. The same compound is formed when heating the *o*-aryloxybenzoic acid with a mixture of acetic anhydride and a small amount of concentrated sulfuric acid.

By sublimation at 130° and 8 μ pressure, and subsequent crystallization of the white sublimate from ethanol (1 Gm. in 25 ml.), the pure 2-benzyl-9-xanthenone is obtained as colorless needles, melting at 135.4–136.1°. It is soluble in benzene, but only slightly in ether. Its solution in concentrated sulfuric acid is yellow, with intense bluish fluorescence under ultraviolet light.

Anal.—Calcd. for $C_{20}H_{14}O_2$: C, 83.89; H, 4.92. Found: C, 84.33; H, 4.79.

2-Benzyl-9-hydroxy-9-phenylxanthene (I).—The Grignard reagent (100% excess) made from 2.43 Gm. magnesium, 15.7 Gm. bromobenzene, and 50 ml. ether was added to a solution of 14.3 Gm. 2-benzyl-9-xanthenone in 120 ml. benzene at 40°. The mixture was refluxed for one hour. After cooling and the addition of water, the solvent was steamed out. The remaining brownish-yellow oil solidified (18.8 Gm., m. p. 100–112°).

For purification, 1 Gm. is dissolved in a mixture of 8 ml. acetic acid and 2 ml. 3 *N* hydrochloric acid, and water added to the yellow filtrate. The oil droplets will solidify on warming (m. p. 118–120°). The hydrol may also be crystallized by adding 3 ml. petroleum ether to a solution of 1 Gm. in 2 ml. benzene.

The pure compound (I) melts at 118–121°. Its solution in concentrated sulfuric acid is of strong yellow color with green fluorescence. It dissolves in concentrated hydrochloric acid as well as in 85% phosphoric acid with reddish-orange color but without fluorescence.

Anal.—Calcd. for $C_{26}H_{20}O_2$: C, 85.71; H, 5.53. Found: C, 85.36; H, 5.64.

2-Benzyl-9-ethoxy-9-phenylxanthene.—When a solution of 6 Gm. of I in 100 ml. of ethanol is refluxed a short time, needles form on cooling. These melt at 80.7–81.7°. Their solution in concentrated sulfuric acid is yellow with green fluorescence.

Anal.—Calcd. for $C_{28}H_{24}O_2$: C, 85.66; H, 6.16. Found: C, 85.11; H, 6.30.

2-Benzyl-9-phenylxanthene.—A mixture of 10 Gm. of hydrol I, 30 Gm. sodium formate, and 80 ml. 99% formic acid (8) was refluxed for one-half hour. The crystalline mass, obtained after the addition of water, was dissolved in 600 ml. ethanol. After evaporation of 450 ml. ethanol from the filtrate, crystals formed. These were then sublimed at 120–125° and 6 μ pressure. The white crystalline sublimate (7.07 Gm.; yield 74%) melted at 122–125°.

Pure 2-benzyl-9-phenylxanthene, obtained as colorless crystals by further crystallization from ethanol (1 Gm. in 55 ml.) melted at 126.2–126.9°. It is insoluble in concentrated sulfuric acid at 25°.

Anal.—Calcd. for $C_{26}H_{20}O$: C, 89.62; H, 5.78. Found: C, 89.36; H, 5.67.

Infrared Spectroscopy

The infrared spectra were evaluated by Dr. B. Katlafsky of Monsanto Chemical Co., St. Louis 24, Mo.

Compound V.—An overall similarity exists between phthalides and compound V, phenolphthalein having the closest, and fluoran the second best resemblance to V. *Ortho* and *para* substitution is found on the phenyl ring. The dilactone structure is substantiated, one carbonyl coinciding with the one of phenolphthalein and the other with the fluoran carbonyl. In the 3 μ section of compound V, there is a single free hydroxyl.

Compound VI.—A study of the infrared spectrum of compound of m. p. 125° shows that it is either an aryl ether or an unsaturated ether of the structure —C=C—O—C— such as occurs in xanthene. The strong bands at 13.2 and 14.4 indicate that *ortho*-disubstituted and -monosubstituted aromatic nuclei

are present. The absence of an absorption band at 3.5 of equal or greater intensity to the aromatic C—H band at 3.3 indicates that no CH_2 or CH_3 groups are present. The spectrum of VI resembles, in many respects, that of 9-phenylxanthene or 9-benzylxanthene.

Compound X.—The infrared spectrum shows a weak band at 11 mμ which is absent in the spectrum of phenolphthalein and of isophenolphthalein. Since we are dealing with aromatic nuclei, this band could be interpreted as the vibration of one isolated ring hydrogen atom situated between substituents. The band for the vibration of such an isolated ring hydrogen atom in 1,2,4 and 1,3,5 trisubstituted and 1,2,3,5 and 1,2,4,5 tetrasubstituted aromatic nuclei is expected to be weak. The spectrum also shows that the *ortho* disubstituted aromatic band at 13.2 increases in intensity with respect to its counterpart in the spectrum of phenolphthalein.

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Dosage Schedule and Pharmacokinetics in Chemotherapy*

By EKKEHARD KRÜGER-THIEMER

The relationships between the chemotherapeutical dosage schedule (supporting dose D, ratio of initial to supporting dose D^+/D, dosing interval τ) and the pharmacokinetical constants k_1, k_2, and V_d, the adsorption constants α and β, and the minimum inhibition concentration μ of the bacteriostatic drug, are formulated.

IN THE CHEMOTHERAPY with sulfanilamides and other bacteriostatic drugs, a therapeutically effective minimum concentration c_{min} of the drug in the blood plasma should be reached as soon as possible and maintained for the duration of the therapy by applying a dosage schedule consisting of an initial dose D^+ and several lower supporting doses D, repeated with dosing intervals τ. In this paper, the functional relationships between the dosage schedule and the plasma concentration c, influenced by the pharmacokinetics, the adsorption by the plasma proteins, and the bacteriostatic activity of the drug, are described. In the following equations there will be used the terms (units in parentheses): t, time after first drug administration (h); τ, dosing interval (h); D/G, relative supporting dose (mg./Kg.); G, body weight of patient (Kg.); D^+/D, dose ratio (mg./mg.); k_1, rate constant of invasion (h^{-1}), k_2, rate constant of elimination (h^{-1}); t_{50}, time of half elimination (h) = $ln2/k_2$; V_d, relative volume of distribution (ml./Gm.); α, drug concentration in plasma ultrafiltrate at half saturation of adsorption (μmol./L); β, maximal specific adsorption by plasma proteins (μmol./Gm.); c plasma concentration of the drug (μmol./L); c_0, extrapolated (fictive) initial concentration in the plasma (μmol./L): c_n, last measured value of concentration in the plasma (μmol./L); c', concentration of freely dissolved drug in plasma water (μmol./L); M, molecular weight of drug (Gm./mol.); w, ratio of volume of plasma water to volume of whole plasma: p, concentration of proteins in blood plasma (Gm./L); μ, minimum inhibition concentration against the infecting bacteria in a liquid medium free from antagonists (μmol./L); σ, proportionality constant in Eq. 1.

The following five assumptions are made: (*a*) According to Davis (1), the bacteriostatic activity of the drug in the blood depends on the concentration c' of the freely dissolved (ultrafiltrable) drug in the plasma water: this concentration c'_{min} necessary for effective therapy should be proportional to the minimum inhibition concentration μ

$$c'_{min} = \sigma \cdot \mu \qquad \text{(Eq. 1)}$$

* Received August 26, 1959, from the Tuberkuloseforschungsinstitut Borstel, Borstel bei Bad Oldesloe, Holstein, Bundesrepublik, Western Germany.
Presented to the session of Advisory Council of the Tuberkuloseforschungsinstitut Borstel, October 31, 1959.
The author is grateful to Dr. Paul Bünger and Werner Diller, Hamburg, Dr. Wilhelm Uecker, Berlin, and Prof. Hans Griem, College Park, Maryland, for critical discussions.

where σ depends on the bacteriostatic, degenerative, or bactericidal character of the drug action and on the content of antagonists in body fluids. A rational method for the determination of σ does not exist; σ may be approximately obtained by comparison of clinical experience with bacteriological results.

(*b*) According to Witzgall (2) after reaching the equilibrium of distribution there will be equal concentrations c' of freely dissolved drug in the plasma water and in the water of all parts of the body, into which the drug may permeate.

$$c' = c_1' = c_2' = c_3' = \ldots \qquad \text{(Eq. 2)}$$

Therefore the conditions of drug action are equal in these parts of the body.

(*c*) The adsorption of the drug by plasma proteins is described by the adsorption isotherm of Langmuir (3), i. e., by the following relationship between the concentration c in the plasma and the concentration c' in the plasma ultrafiltrate of the bacteriostatic form of the drug

$$c = c' \cdot \left(w + \frac{\beta \cdot p}{\alpha + c'} \right) \qquad \text{(Eq. 3)}$$

(*d*) The time-plasma concentration relationship is described with sufficient accuracy according to Dost (4) by the equation

$$c = \frac{c_o \cdot k_1}{k_1 - k_2} \cdot \left(e^{-k_2 t} - e^{-k_1 t} \right) \qquad \text{(Eq. 4)}$$

(*e*) According to Dost (4) and Boxer, *et al.* (5), the fictive initial concentration c_o is a function of D. The ratio of these two terms is called volume of distribution and, after division by the body weight G, relative volume of distribution V_d

$$V_d = \frac{D}{c_o} \cdot \frac{1000}{G \cdot M} \qquad \text{(Eq. 5)}$$

In the usually applied range of doses of several sulfanilamides V_d is found to be a constant (6).

Dost (4) has given equations for the accumulation of a drug applied in several equal doses D at constant intervals τ. Earlier approaches to this problem were made by Widmark and Tandberg (7), by Boxer, *et al.* (5), and by Druckrey and Küpfmüller (8). From Dost's equation for the lower limit of accumulation

$$c_{min} = \frac{c_o \cdot k_1}{k_1 - k_2} \cdot \left(\frac{1}{1 - e^{-k_2 \tau}} - \frac{1}{1 - e^{-k_1 \tau}} \right) \qquad \text{(Eq. 6)}$$

and from the above mentioned five assumptions the following equations for the dose ratio with extravasal application

$$\frac{D^+}{D} = \frac{1}{(1 - e^{-k_1 \tau}) \cdot (1 - e^{-k_2 \tau})} \qquad \text{(Eq. 7)}$$

and for the relative supporting dose D/G

$$\frac{D}{G} = \frac{M \cdot \sigma \cdot \mu}{1000} \cdot \left(w + \frac{\beta \cdot p}{\alpha + \sigma \cdot \mu} \right) \cdot V_d \cdot \left(1 - \frac{k_2}{k_1} \right) \cdot \frac{(1 - e^{-k_1 \tau}) \cdot (1 - e^{-k_2 \tau})}{e^{-k_2 \tau} - e^{-k_1 \tau}} \qquad \text{(Eq. 8)}$$

can be derived. Equation 7 is consistent within the range of doses having constant values of V_d (see Eq. 5). For intravenous application with $k_1 \to \infty$, the equations of the dosage schedule are

$$\frac{D^+}{D} = \frac{1}{1 - e^{-k_2 \tau}} \qquad \text{(Eq. 9)}$$

and

$$\frac{D}{G} = \frac{M \cdot \sigma \cdot \mu}{1000} \cdot \left(w + \frac{\beta \cdot p}{\alpha + \sigma \cdot \mu} \right) \cdot V_d \cdot (e^{k_2 \tau} - 1) \qquad \text{(Eq. 10)}$$

The equations for continuous intravenous infusion result from Eqs. 7 and 8 by $k_1 \to \infty$ and $\tau \to 0$ with D/t being the infusion rate

$$\frac{D^+}{D/t} = \frac{1}{k_2} \qquad \text{(Eq. 11)}$$

$$\frac{D}{G \cdot t} = \frac{M \cdot \sigma \cdot \mu}{1000} \cdot \left(w + \frac{\beta \cdot p}{\alpha + \sigma \cdot \mu} \right) \cdot V_d \cdot k_2 \qquad \text{(Eq. 12)}$$

The values of k_1, k_2, V_d, α and β, which are necessary for the calculation of D^+/D and D/G, may be obtained from any complete time-plasma concentration curve determined after a single dose D of the drug within the supposed therapeutically effective dose range, and from two (or better, more) estimates of the plasma ultrafiltrate concentration c' in relation to the plasma concentration c over a concentration range of 1 to 10. k_2 is equal to the negative slope of the descending straight part of the time-log$_e$ c curve [Dost (4), Boxer, *et al.* (5), Swintosky, *et al.* (9)]. For the calculation of V_d from Eq. 5 and of k_1 from Eq. 4, the value of c_o corresponding to the applied relative dose D/G must be determined. For this purpose, a relationship, recently found by Dost (10), may be used which connects the area F between the time-plasma concentration curve and the abscissa axis with c_o and k_2

$$F = \int_o^\infty c \cdot dt = \frac{c_o}{k_2} \qquad \text{(Eq. 13)}$$

This follows from Eq. 4 by integration. If, at the end of the experiment ($t = t_n$), the ordinate c_n is not negligible, the integration of the curve from 0 to t_n does not yield the whole integral of Eq. 13. As the deficient part of the area equals c_n/k_2 in good approximation, c_o may be calculated from the equation.

$$c_o = k_2 \cdot \int_o^{t_n} c \cdot dt + c_n \qquad \text{(Eq. 14)}$$

the integral in which may be obtained planimetrically or by calculation using the formula of sums of trapezes. k_1 may be gained from Eq. 4 by series evolution for $e^{-(k_1-k_2)t}$, whereby three approximations may be calculated, explicitly, the first and second of which are

$$k_1' = \frac{\frac{c}{t}}{c_0 \cdot e^{-k_2 t}} \qquad \text{(Eq. 15)}$$

$$k_1'' = \frac{1}{t} \cdot \left(1 + \frac{k_2 \cdot t}{2} \pm \sqrt{\left(1 + \frac{k_2 \cdot t}{2}\right)^2 - 2 \cdot k_1' \cdot t}\right) \qquad \text{(Eq. 16)}$$

It is of interest, that the influence of k_1, which is quite variable at peroral application, on Eqs. 7 and 8 is small. Therefore, in most cases, the first approximation (Eq. 15) calculated with that one of the first measured values, which gives the greatest value of c/t, is sufficient. The two constants of adsorption α and β may be calculated from many pairs of values c_t, c_t', using transformed Eq. 3 with Gauss' method of least squares of errors; in the graph of the straight line, α is given by the negative intercept with the ordinate axis and β is given by the slope of the line (abscissa $= p/[(c/c') - w]$ and ordinate $= c'$).

Before D^+/D and D/G can be calculated, a suitable value of τ has to be selected. For clinical convenience, only proper fractions of twenty-four hours should be taken. From an investigation of many sulfanilamide derivatives (6), it may be mentioned that D^+/D in the clinic is often taken as 2.0. In most of these cases, τ nearly equals the time of half elimination t_{50}. This relation which was found empirically follows also from Eq. 7 for $\tau = t_{50}$, and a great value of k_1. Therefore, it may be stated that the suitable value of the dosing interval τ should be near to the value of the time of half elimination t_{50} of the drug; in this case, the dose ratio D^+/D nearly equals 2.0. Only the relative supporting dose D/G remains to be calculated numerically from Eq. 8. Nomograms for these calculations will be published elsewhere (11). For the calculation of D/G, the value of σ is necessary. As already mentioned, an independent method for the determination of σ is not available. By comparison of clinically used and calculated relative supporting doses D/G a value of $\sigma = 2$ was found for *Escherichia coli* and several sulfanilamide derivatives (11).

The upper limit of the relative supporting dose D/G is given as the toxicity of the drug. Current definitions of the chronic toxicity do not take into consideration the pharmacokinetics of the drug, so that it seems to be necessary to define a new criterion of chronic toxicity: Dosis sustinentis tolerata 95 per cent (DST_{95}, mg./Kg.) is defined as the supporting dose of a dosage schedule (with $D^+/D = 2.0$ and $\tau = t_{50}$), which is tolerated without toxic symptoms by 95 per cent of the treated individuals; the method for estimation of DST_{95} is analogous to that described by Wagner (12) for the dosis curativa 95 per cent (DC_{95}).

From the presented pharmacokinetical theory of the dosage schedule it may be concluded that a full description of the chemotherapeutical properties of a drug requires at least eight values: the three pharamacokinetical constants k_1, k_2, and V_d; the two adsorption constants, α and β; the minimum inhibition concentration μ; the factor σ, and the dosis sustinentis tolerata 95% DST_{95}. These constants may achieve clinical significance only in association with their standard deviations obtained from many (10 or more) patients or experimental animals. The possibility of calculating D^+/D and D/G by Eqs. 7 to 12 is very restricted by the difficulties of the estimation of σ and DST_{95}. In the author's opinion, the main use of these equations is to clarify the mathematical structure of the interrelationships between the properties and the therapeutical effect of a drug, whereby the number of the biological constants necessary for the description of these interrelationships is determined. These biological constants will be useful in comparing various drugs with an analogous mode of action. An application of this pharmacokinetical theory of dosage schedule on new sulfanilamide derivatives (6) and a detailed description of the implications of this theory (11) will be published elsewhere.

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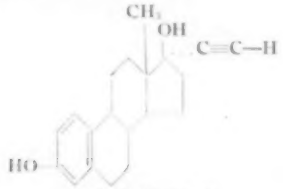
Spectrophotometric Assay for Combinations of Ethinyl Estradiol and Methyltestosterone*

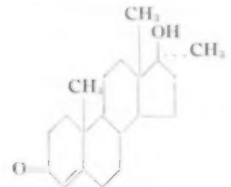
By SHELDON KLEIN, ARTHUR E. JAMES, and MURRAY M. TUCKERMAN

The ultraviolet absorption curves of ethinyl estradiol and of methyltestosterone were determined in acidic and alkaline methanol. The methyltestosterone absorption curve is independent of pH in the range studied; however the absorption of ethinyl estradiol is affected by changes in the pH of the solvent. Ethinyl estradiol has a stable absorption maximum at 281 mμ in acidic methanol and a stable maximum at 298 mμ in alkaline methanol solutions greater than 0.2 *N* in potassium hydroxide. At ratios of ethinyl estradiol to methyltestosterone in the order of 6 : 1 to 1 : 4, simultaneous determination of the two is satisfactory. In commercial products in which the ratio is of the order 1 : 200 to 1 : 500, methyltestosterone can be determined directly, whereas acceptable results for ethinyl estradiol appear to require separation.

METHYLTESTOSTERONE has been included in the U. S. P. since 1947, ethinyl estradiol since 1955. The objective of the work described herewith was to develop a simultaneous assay for these two hormones. Their structural formulas may be represented as shown:



Ethinyl Estradiol



Methyltestosterone

For the identification and the quantitative analysis of ethinyl estradiol, fluorescence, colorimetric, ultraviolet, and infrared measurements have been suggested (1–5). For methyltestosterone, colorimetric, ultraviolet, polarographic, and angular rotation measurements have been used (2, 6–16).

* Received August 21, 1959, from Temple University, School of Pharmacy, Philadelphia 40, Pa.

Based, in part, upon a thesis presented by Sheldon Klein to the Faculty, Temple University, School of Pharmacy, in partial fulfullment of the requirements for the degree of Master of Science in Pharmacy.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

The authors wish to thank the Schering Corp., Bloomfield, N. J.; Ciba Pharmaceutical Products, Inc., Summit, N. J.; and Warren-Teed Products Co., Columbus, Ohio, for their generous supply of products and information for this work. Also, E. R. Squibb and Sons, Division of Olin Mathieson Chemical Corp., New Brunswick, N. J.; Chicago Pharmacal Co., Chicago, Ill.; and Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N. Y., for their generous supply of products used for this work.

EXPERIMENTAL

Absorptivity of Methyltestosterone.—Measurements of U. S. P. Reference Standard methyltestosterone absorption in methanol 1 *N* and 0.01 *N* in hydrochloric acid, and in methanol 0.1 *N*, 0.3 *N*, and 1 *N* in potassium hydroxide, showed that the absorption curve was unaffected by the pH of the solvent. The absorption maximum is at 241 mμ. At this wavelength, absorption follows Beer's law over the range 2 to 20 mcg./ml.; $E(1\%, 1\text{ cm.}) = 508$. This value is not in accord with the U. S. P. figure of 520–540 for an ethanolic solution. At 298 mμ Beer's law is followed in the concentration range 1 to 5 mg./ml.; $E(1\%, 1\text{ cm.}) = 3.30$. The absorption curves are shown in Figs. 1 and 2. The absorptivity values, $E(1\%, 1\text{ cm.})$ are shown in Tables I and II. In this and subsequent work each value is the average of two or more determinations.

TABLE I.—ABSORPTIVITY OF METHYLTESTOSTERONE IN 0.01 *N* HCl-CH_3OH AT 241 mμ

Concentration Methyltestosterone, mcg./ml.	Absorbance at 241 mμ	Absorptivity $E(1\%, 1\text{ cm.})$
2.00	0.102	510
5.00	0.252	504
10.00	0.509	509
20.00	1.020	510
	Average	508

TABLE II.—ABSORPTIVITY OF METHYLTESTOSTERONE IN 0.3 *N* KOH-CH_3OH AT 298 mμ

Concentration Methyltestosterone, mg./ml.	Absorbance at 298 mμ	Absorptivity $E(1\%, 1\text{ cm.})$
1.00	0.315	3.15
2.00	0.660	3.30
5.00	1.653	3.31
	Average	3.25

Absorptivity of Ethinyl Estradiol.—In acid methanol the absorptivity maximum of U. S. P. ethinyl estradiol is at 281 mμ; $E(1\%, 1\text{ cm.}) = 68.6$. Beer's law is followed over the concentration range 10.5 to

107 mcg./ml. In alkaline methanol, the absorption maximum shifts gradually from 281 mμ to 298 mμ as the concentration of potassium hydroxide is increased, until a stable absorption maximum is reached at a concentration about 0.2 *N* potassium hydroxide. At potassium hydroxide concentrations between 0.2 *N* and 1 *N* the absorption maximum is at 298 mμ; $E(1\%, 1\text{ cm.}) = 90$. Absorption curves are shown in Figs. 1 and 2. The absorptivity values are shown in Tables III and IV. The data for the absorptivity of ethinyl estradiol is given at 241 mμ this being the maximum absorption of methyltestosterone.

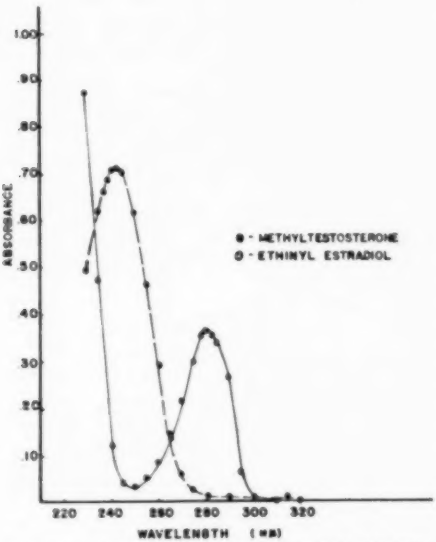


Fig. 1.—Absorption curves in 1*N* HCl-CH_3OH.

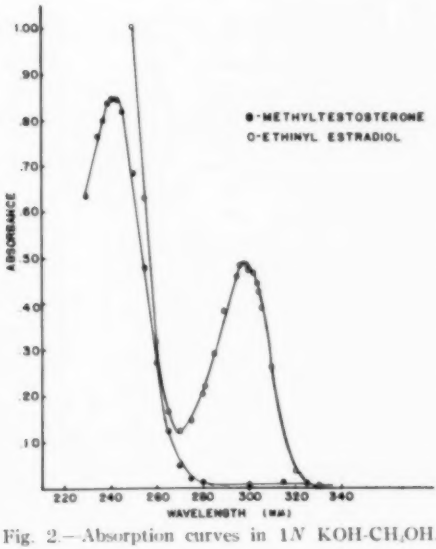


Fig. 2.—Absorption curves in 1*N* KOH-CH_3OH.

TABLE III.—ABSORPTIVITY OF ETHINYL ESTRADIOL IN 0.01 *N* HCl-CH_3OH AT 241 mμ

Concentration Ethinyl Estradiol, mcg./ml.	Absorbance at 241 mμ	Absorptivity $E(1\%, 1\text{ cm.})$
18.8	0.034	18.0
37.5	0.068	18.0
75.0	0.132	17.6
	Average	18.0

TABLE IV.—ABSORPTIVITY OF ETHINYL ESTRADIOL IN VARIOUS ALKALINE METHANOL SOLUTIONS AT 298 mμ

Solvent	$E(1\%, 1\text{ cm.})$
0.01 *N* KOH-CH_3OH	56.0
0.05 *N* KOH-CH_3OH	86.0
0.1 *N* KOH-CH_3OH	88.0
0.2 *N* KOH-CH_3OH	90.2
0.3 *N* KOH-CH_3OH	90.4
0.5 *N* KOH-CH_3OH	90.6

Standard Series.—A series of mixtures in chloroform was prepared in which the ratio of the concentrations of ethinyl estradiol to methyltestosterone was varied from 6:1 to 1:1. Aliquots were evaporated and taken up in methanol 0.01 *N* in hydrochloric acid and in methanol 0.3 *N* in potassium hydroxide. Another series was prepared by separately dissolving the hormones in methanol. Aliquots of each solution were taken to prepare a series of mixtures containing a ratio of concentrations of ethinyl estradiol to methyltestosterone from 1:1 to 1:5. One aliquot of each mixture was diluted with methanol 0.01 *N* in hydrochloric acid, a second, with methanol 0.3 *N* in potassium hydroxide.

Simultaneous Spectrophotometric Determination of Standards.—The absorbance of the acidic solutions was measured at 241 mμ; that of the basic solutions at 298 mμ, see Table V. The concentrations of each hormone were calculated by the method of simultaneous equations (17). These equations reduce to the following forms:

$$\text{concentration of ethinyl estradiol (mg./ml)} = (51\,A_{298} - 0.33\,A_{241})/458$$

and concentration of methyltestosterone (mg./ml.) = $A_{241} - 1.8 \times$ concentration of ethinyl estradiol (mg./ml.)/51.

A refers to the measured absorbance of the solution, the subscript denotes the wavelength at which the measurement was made. All measurements were made in a cell with a path of 1 cm.

Assay of Commercial Products for Methyltestosterone.—Current pharmaceutical products containing mixtures of ethinyl estradiol and methyltestosterone in the order of 1:200 to 1:500 were assayed for methyltestosterone without separation of the hormones. Aliquots of powdered tablets equivalent to 120 mg. of testosterone were extracted with three successive portions of ether (U. S. P.) The combined ether extracts were washed with three successive 10-ml. portions of 10% $NaHCO_3$, followed by two successive 10-ml. portions of 0.1 *N* HCl. The ether extract was made up to a volume of 100 ml. with this solvent. Two-milliliter portions of the

TABLE V.—SIMULTANEOUS ASSAY OF KNOWN MIXTURES OF ETHINYL ESTRADIOL AND METHYLTESTOSTERONE IN 0.01 *N* HCl-CH_3OH AND 0.3 *N* KOH-CH_3OH

Solution	Ethinyl Estradiol			Methyltestosterone		
	Theoretical, mcg./ml.	Experimental mcg./ml.	Error, %	Theoretical, mcg./ml.	Experimental, mcg./ml.	Error, %
A	30.0	29.9	−0.3	4.88	4.57	−6.3
B	45.0	45.2	+0.4	9.76	9.57	−2.0
C	15.0	15.2	+1.3	4.96	4.92	−0.8
D	15.0	15.0	0.0	9.92	9.75	−1.7
E	15.6	15.3	+2.0	12.2	11.8	−3.3
F	5.11	5.04	−1.4	5.00	5.00	0.0
G	5.11	5.30	+3.7	10.0	9.98	−0.2
H	5.11	5.15	+0.8	20.0	19.8	−1.0
I	5.11	5.76	+12.7	25.0	24.6	−1.6

ether solution were evaporated to dryness. The residue was taken up in methanol 0.1 *N* in HCl and made up to a volume of 250 ml. The absorbance of this solution was determined at 241 mμ. The methyltestosterone in mg./ml. = $A_{241}/50.8$.

Suitable volumes of liquid preparations containing the two hormones in the above-mentioned ratios were extracted with ether. The combined ether extracts were washed with distilled water and made up to a volume of 100 ml. with ether. Two-milliliter aliquots were evaporated to dryness and the residue taken up in 250 ml. of methanol 0.1 *N* in HCl. The absorbance of the resulting solutions was measured at 241 mμ and the methyltestosterone content calculated by the same formula used for tablets. Typical results are shown in Table VI.

TABLE VI.—METHYLTESTOSTERONE IN COMMERCIAL PRODUCTS

Product	Labelled Content	Experimental	Deviation, %
Tablets A	10 mg./tablet	9.6	4.0
Tablets B	5 mg./tablet	4.5	10.0
Liquid X	2.5 mg./ml.	2.3	8.0

DISCUSSION

The simultaneous spectrophotometric assay is based on the absorption of solutions in acidic and alkaline methanol. Though the absorption curve of methyltestosterone appears stable throughout the pH range investigated, the absorption curve for ethinyl estradiol depends upon the degree of ionization of its phenolic group. This group appears to be unionized in acidic methanol and to be completely ionized in alkaline methanol greater than 0.2 *N* in potassium hydroxide.

Methyltestosterone is calculated from the absorbance in acidic methanol in order to minimize interference from ethinyl estradiol. Ethinyl estradiol is calculated from the absorbance in alkaline methanol in order to minimize interference from methyltestosterone and to increase the sensitivity of the assay, since ethinyl estradiol has its absorption maximum at a longer wavelength and has a higher absorbance in alkaline methanol than in acidic methanol.

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Some Ketonic Mannich Bases*

By ELIZABETH D. TAYLOR and W. LEWIS NOBLES

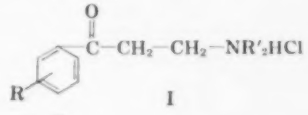
A group of ketonic Mannich bases, represented by Types I and II, were synthesized for pharmacological evaluation. Some of these agents demonstrated *in vitro* antitubercular and amebicidal action.

INTEREST HAS BEEN EXPRESSED in the pharmacological action of various ketonic Mannich bases (1–8). The extensive literature dealing with this reaction has been reviewed recently by Reichert (9).

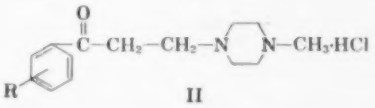
Mannich and Lammering reported that β-piperidinopropiophenone hydrochloride possessed local anesthetic activity (1); it was observed by others (2) that the similar amino analog from 2-acetylpyrrole demonstrated this same type of activity. It has been indicated by Levvy and Nisbet (3) that both the piperidino and dimethylamino Mannich bases of 2-acetylthiophene possessed local anesthetic activity; subsequently, Denton and his associates (4) described the antispasmodic activity of compounds of this type and other closely related structures.

Burckhalter and Johnson (5) reported the antibacterial activity of certain Mannich bases derived from α,β-unsaturated ketones. We have previously reported analogs of some of these more active compounds (6). Mercier and his associates indicated that the diethylamino Mannich base from acetophenone was adrenolytic, hypotensive and ganglioplegic (7). More recently, Issekutz and co-workers (8) reported that certain substituted propiophenones of this type inhibited nicotine tremors and convulsions in rabbits, and raised the lethal dose of nicotine in mice. The 3-diethylamino compound was the most potent compound reported in this study; in addition to this type of activity, this compound inhibited water diuresis in rats when administered at a dosage level of 5 mg./100 Gm. body weight. Several of the agents reported in this study were weak spasmolytic agents when tested on isolated rabbit and guinea pig intestinal strips.

In view of this multiplicity of pharmacological effects elicited by these simple ketonic Mannich bases, we wished to prepare a number of previously unreported Mannich bases of Type I, especially those containing halogen substituents in view of the well known effect of halogen substituents increasing activity in many compounds demonstrating these general types of pharmacological activity. In addition, the recent interest in the pharmacological activity of piperazine and its derivatives prompted us to prepare compounds of Type II, in which the amine moiety is the disubstituted piperazine.

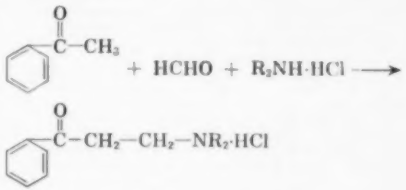


I



II

The preparations of compounds of Types I and II, which are listed in Tables I and II, was accomplished by means of the Mannich reaction, which involved the interaction of formaldehyde or paraformaldehyde with the appropriate ketone and amine hydrochloride. This reaction may be depicted as follows:



EXPERIMENTAL

Experimental Results.—*p*-Fluoroacetophenone and *p*-iodoacetophenone were prepared according to the method of Lutz (10). *p*-Chlorobenzalacetone was synthesized according to the method previously outlined (11). The N-methylpiperazine was kindly supplied through the courtesy of Dr. Harold Zaugg of Abbott Laboratories. All the other ketones and amines used in this study were commercially available.

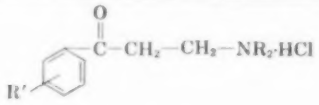
The Mannich reaction was carried out as previously described (6) utilizing the two procedures therein alluded to depending upon the physical state in which the amine or its hydrochloride was usually available. In general, the reactions proceeded smoothly to yield the expected product. It may be noted, however, that excellent yields are seldom obtained in the Mannich reaction due to the complexity of the products obtained which may be occasioned by by-product formation.

* Received August 21, 1959, from the University of Mississippi, School of Pharmacy, University.

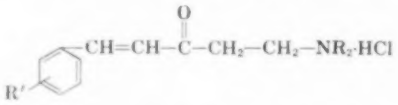
This paper was based on a portion of a thesis submitted by Mrs. Elizabeth D. Taylor in partial fulfillment of the requirements for the Master of Science degree in the Graduate School of the University of Mississippi, August 1955.

Presented to the Scientific Section A. PH. A., Cincinnati meeting, August 1959.

TABLE I.—MANNICH BASES



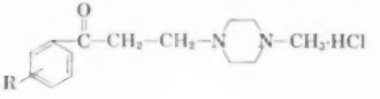
No.[a]	R′	NR_2	Yield, %	M. P.,[b] °C.	Formula	Analyses, [c] % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1.	*p*-Fluoro	Dimethylamino	48	156–158	$C_{11}H_{14}FNO \cdot HCl$	57.00	57.01	6.52	6.39
2.	*p*-Fluoro	*n*-Dibutylamino	42	189–190	$C_{17}H_{26}FNO \cdot NCl$	64.64	64.83	8.61	8.49
3.	*p*-Fluoro	Piperidino	68	190	$C_{14}H_{18}FNO \cdot HCl$	61.87	61.63	7.05	7.45
4.	*p*-Fluoro	Morpholino	65	215–217	$C_{13}H_{16}FNO_2 \cdot HCl$	57.04	56.80	6.26	6.16
5.	*p*-Fluoro	(2-Methyl)-piperidino	22	205–206	$C_{15}H_{20}FNO \cdot HCl$	63.26	62.81	7.43	7.23
6.	*p*-Fluoro	Pyrrolidino	38	145	$C_{13}H_{16}FNO \cdot HCl$	60.58	60.30	6.65	7.08
7.	*p*-Iodo	Dimethylamino	42	208–210	$C_{11}H_{14}INO \cdot HCl$	38.90	38.96	4.45	4.61
8.	*p*-Iodo	Piperidino	52	214–216	$C_{14}H_{18}INO \cdot HCl$	44.28	44.20	5.04	5.16
9.	*p*-Iodo	Morpholino	47	209–210	$C_{13}H_{16}INO_2 \cdot HCl$	40.91	40.87	4.49	4.61
10.	*p*-Iodo	(2-Methyl)-piperidino	26	209–210	$C_{15}H_{19}INO \cdot HCl$	45.87	45.63	5.39	5.22
11.	*p*-Iodo	Pyrrolidino	34	199–200	$C_{13}H_{16}INO \cdot HCl$	42.70	42.76	4.68	4.79
12.	*o*-Hydroxy[d]	Dimethylamino	33	175–176	$C_{11}H_{15}NO \cdot HCl$	...	...	...	...
13.	*o*-Hydroxy	Piperidino	65	180	$C_{14}H_{19}NO_2 \cdot HCl$	62.33	61.71	7.49	7.38
14.	*o*-Hydroxy	Morpholino	59	193–195	$C_{13}H_{17}NO_3 \cdot HCl$	57.45	57.31	6.68	6.49
15.	*o*-Hydroxy	(2-Methyl)-piperidino	57	210–212	$C_{15}H_{21}NO_2 \cdot HCl$	63.48	62.95	7.81	7.68
16.	*o*-Hydroxy	Pyrrolidino	28	161–162	$C_{13}H_{17}NO_2 \cdot HCl$	61.05	60.59	7.09	7.28



17.	*p*-Chloro	Piperidino	69	198–200	$C_{14}H_{16}ClNO \cdot HCl$	61.15	61.35	6.73	6.58
18.	*p*-Methoxy	(2-Methyl)-piperidino	75	212–213	$C_{16}H_{23}NO_2 \cdot HCl$	66.75	66.28	8.12	8.34

[a] All Mannich bases in this table were recrystallized from an ethanol-acetone solution.
[b] Melting points are uncorrected.
[c] Carbon and hydrogen analyses are by Weiler and Strauss, Oxford, England.
[d] Padfield, E. M., and Tomlinson, M. L., *J. Chem. Soc.*, **1950**, 2272, report a m. p. of 176° for a compound which they indicate to be the dimethylamino Mannich base, although in their description they refer to the use of methylamine. The nature of their investigation would dictate that it was, in fact, the dimethylamino compound they were utilizing in connection with the work reported in the reference cited.

TABLE II.—MANNICH BASES



No.	R	Yield, %	M. P.,[a] °C.	Formula	Analyses, [b] % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1.	*p*-Fluoro	78	181	$C_{14}H_{19}FN_2O \cdot 2HCl \cdot H_2O$	49.27	49.86	6.79	6.54
2.	*p*-Chloro	80	179–180	$C_{14}H_{19}ClN_2O \cdot 2HCl \cdot H_2O$	47.00	46.69	6.48	6.61
3.	*p*-Bromo	68	185–187	$C_{14}H_{19}BrN_2O \cdot 2NCl \cdot 3H_2O$	38.39	38.82	6.21	6.18
4.	*p*-Iodo	77	176–177	$C_{14}H_{19}IN_2O \cdot 2HCl \cdot 4H_2O$	33.44	33.48	5.81	6.05
5.	None[c]	58	180–181	$C_{14}H_{20}N_{20} \cdot 2HCl \cdot H_2O$	52.01	52.10	7.48	7.16
6.	*p*-Nitro	71	192–194	$C_{14}H_{19}N_3O_3 \cdot 2HCl \cdot H_2O$	45.66	45.93	6.29	6.41
7.	*p*-Methoxy	81	190–192	$C_{15}H_{22}N_2O_2 \cdot 2HCl \cdot 2H_2O$	48.29	48.43	7.56	7.60
8.	*p*-Ethoxy	70	187–188	$C_{16}H_{24}N_2O_2 \cdot 2HCl \cdot H_2O$	52.32	52.51	7.68	7.40
9.	*m*-Nitro	38	170	$C_{14}H_{19}N_3O_3 \cdot 2HCl \cdot H_2O$	45.66	45.38	6.29	6.16

[a] Melting points are uncorrected.
[b] Carbon and hydrogen analyses are by Weiler and Strauss, Oxford, England.
[c] Cymerman-Craig, J., and Harrison, R. J., *Australian J. Chem.* **8**, 378(1955), refer to an anhydrous form of this compound which melts at 197°; this reference was obtained from *Chem. Abstr.* after the completion of the present work. Denton, J. J., *et al.*, *J. Am. Chem. Soc.*, **71**, 2048(1949), report a similar compound with 0.75 mole of water of hydration for which they report a melting point of 187.1–188.5°. It is of interest that all of the present compounds were obtained as hydrates when the usual Mannich procedure was used with recrystallization from ethanol-acetone.

TABLE III.—TUBERCULOSIS SCREENING, RESULTS *in Vitro*, SERUM BROTH DILUTION

	Compound	Organism	Strain	Incub., Days	Mcg./ml. Causing Complete Inhibition	Partial Inhibition	Standard Control (INH) mcg./ml. Causing Complete Inhibition
1.	No. 1	*M. tuberculosis*	...	Inactive	..	..	...
2.	No. 9	*M. tuberculosis*	...	Inactive	..	..	...
3.	No. 10	*M. tuberculosis*	H37RV	7	20	..	0.024
4.	No. 10	*M. tuberculosis*	INH-Res.	7	20	..	...
5.	No. 10	*M. tuberculosis*	STM-Res.	7	20	..	...
6.	No. 13	*M. tuberculosis*	H37RV	7	20	..	0.024
7.	No. 13	*M. tuberculosis*	INH-Res	7	20	10	...
8.	No. 13	*M. tuberculosis*	STM-Res.	7	20	10	...

TABLE IV.—AMEBICIDAL ACTIVITY

Compound	Dilution	Drug Effect
No. 1	1:2500	Cidal
No. 1	1:5000	Cidal
No. 9	1:5000	Cidal
No. 13	1:2500	Cidal
No. 13	1:5000	Stasis

Pharmacological Results.—The results of the pharmacological evaluation of these compounds have been made available through the courtesy of Dr. Loren Long of Parke, Davis and Co. All of the compounds thus far tested are from Table I and the numbers used in this section will refer to the numbers cited in that table only. The *in vitro* antitubercular and amebicidal activities of some of these compounds are listed in Tables III and IV.

Despite the *in vitro* activity of compounds No. 10 and 13 in the tuberculosis screening test, these compounds were regarded as being inactive in subsequent *in vivo* tests. Also, compound No. 9 was not regarded as being promising in subsequent *in vivo* amebicidal tests which were conducted. Compound No. 11 did not demonstrate any *in vivo* activity against either *Strep. pyrogenes* or *Staph. aureus*.

Moreover, compound No. 1 evidenced no respiratory stimulation in doses ranging from 2–32 mg./Kg. when administered intravenously to cats. No cerebral stimulation was noted with this compound in doses up to 100 mg./Kg. The same negative results were elicited by compounds No. 8 and 13. A mild degree of respiratory stimulation was elicited, however, by compound No. 9 when it was administered to cats at a dosage level of 4–8 mg./Kg., but no cerebral stimulation was evidenced with this agent at doses up to 100 mg./Kg.

Compound No. 1 produced stasis in *in vitro* tests against *Trichimonas vaginalis* at a concentration of 50 mcg./ml. but was inactive at a level of 12.5 mcg./ml.

SUMMARY

Twenty-seven Mannich bases have been prepared using various ketones with dialkyl and heterocyclic amines. While several of these compounds did demonstrate *in vitro* activity against various organisms as reported in this paper, subsequent *in vivo* tests of these substances were not regarded as promising in any instance.

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Quantitative Determination of Ethanol in Pharmaceutical Products by Gas Chromatography*

By HAROLD J. WESSELMAN

The presently used U. S. P. XV method for ethanol is compared with the gas chromatography method. Advantages and disadvantages of both methods are discussed. Typical results obtained by both procedures when applied to tinctures, fluidextracts, elixirs, and other products are compared. The results obtained from three gas chromatographs each having a different type thermal conductivity detector are presented. Three types of sample injectors are compared and five different stationary phases are examined.

SINCE MANY pharmaceutical preparations contain ethanol, it is desirable to use a method for the determination of ethanol which is both fast and accurate. The presently used distillation method described in the U. S. P. XV (1) is time consuming, at least one hour per assay, and often complicated by frothing and bumping during the distillation. Samples containing glycerin, iodine, volatile oils, solvents, or ammonia require special treatment prior to distillation. The determination of the specific gravity of the distillate offers another possibility for error.

The successful application of gas chromatography in previous work (2) suggested that it might be used for ethanol. Preliminary work showed that it offers an ideal solution to the above mentioned problems as samples can be assayed with little or no treatment in a short time with equal or better accuracy.

EXPERIMENTAL

Stationary Phase.—In the search for a suitable stationary phase polyethylene glycol 400, diglycerol, dinonyl phthalate, Ucon 75-H-90000, and tricresyl phosphate were examined. Polyethylene glycol 400 was finally selected since it resolves ethanol, water, and acetone completely and the retention times are short, an important factor when many samples are to be assayed.

Sample Injector.—Initially, the peak height of ethanol in the sample being assayed was compared to the peak height of a standard ethanol solution. Good results were obtained, but the problem of repetitive injection of small samples (1 to 5 μL.) was very tedious.

The Hamilton microliter syringe No. 705N proved to be the most useful of the three injectors available since it is easy to use and to clean. The Agla microliter syringe was satisfactory but requires time to assemble, load, inject sample, disassemble, and clean. The final system used was that of Tenney and Harris (3). This system proved satisfactory although some liquids are difficult to load into the capillary pipets. Each of these injectors gave comparable results.

* Received August 21, 1959, from the Analytical Control Research and Development Departments, Eli Lilly and Co., Indianapolis, Ind.
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

TABLE I.—RATIO OF PEAK HEIGHTS OF ETHANOL AND ACETONE FOR STANDARD SOLUTIONS OF ETHANOL AND ACETONE

Preparation	Ethanol, %	Acetone, %	Ratio Ethanol / Acetone
Elixir Betalin[a] complex	13	10	1.04
Elixir Betalin[a] complex	14	10	1.10
Elixir Betalin[a] complex	15	10	1.15
Elixir Betalin[a] complex	16	10	1.21
Elixir Betalin[a] complex	17	10	1.27
F. E. Cascara sagrada aromatic	12	10	1.15
F. E. Cascara sagrada aromatic	14	10	1.23
F. E. Cascara sagrada aromatic	16	10	1.31
F. E. Cascara sagrada aromatic	18	10	1.39
F. E. Cascara sagrada aromatic	20	10	1.47
Elixir Amytal[b]	24	20	0.935
Elixir Amytal[b]	26	20	0.993
Elixir Amytal[b]	28	20	1.055
Elixir Amytal[b]	30	20	1.114
Elixir Amytal[b]	32	20	1.171
Fluid rose, soluble	30	30	0.7512
Fluid rose, soluble	32	30	0.7850
Fluid rose, soluble	34	30	0.8242
Fluid rose, soluble	36	30	0.8653
Fluid rose, soluble	38	30	0.9000
Tincture belladonna	44	30	1.026
Tincture belladonna	46	30	1.060
Tincture belladonna	48	30	1.104
Tincture belladonna	50	30	1.133
Tincture belladonna	52	30	1.171
Spirit camphor	70	10	4.125
Spirit camphor	72	10	4.177
Spirit camphor	74	10	4.250
Spirit camphor	76	10	4.275
Spirit camphor	78	10	4.324

[a] Betalin complex, vitamin B complex, Lilly.
[b] Amytal, amobarbital, Lilly.

Calibration.—Since the accuracy of the direct measurement of peak heights for quantitative determinations of ethanol depends mainly on the ability to inject accurate volumes of samples, this point was given serious consideration. The internal normalization technique cannot be applied because of the numerous volatiles and nonvolatiles found in the samples encountered. The internal standard technique introduced by Ray (4) and later used by Bradford, Harvey, and Chalkley (5) was then used since it is the most accurate of all methods, ±1% being easily achieved. In this method a suitable

TABLE II.—COMPARISON OF ETHANOL DETERMINATIONS BY DISTILLATION AND GAS CHROMATOGRAPHY

Preparation	Theory, %	Distillation, %	Gas Chromatography, %			
			Analyst 1	Analyst 2	Average	Standard Deviation
Elixir Betalin complex	17.00	17.27	16.90	17.33		
		16.83	17.16	16.66		
		16.67	17.77	17.78	17.27	0.97
F. E. Cascara sagrada aromatic	18.00	18.00	18.18	17.77		
			17.90	17.85		
			17.50	17.50	17.78	0.52
Elixir Amytal	34.00	32.88	33.55	33.33		
			34.22	33.60		
			34.10	34.20	33.83	0.91
Fluid rose, soluble	48.00	49.08	48.88	47.77		
			49.55	48.57		
			48.00	48.71	48.58	1.25
Tincture belladonna U. S. P.	67.00	67.14	66.66	68.57		
			68.43	66.11		
			68.40	67.14	67.55	2.45
Spirit camphor N. F.	83.00	86.00	85.33	82.11		
			82.44	81.88		
			83.15	84.64	83.26	3.70

volatile substance is added to the sample in known proportion and the peak height of the ethanol is referred to the peak height of the internal standard. Calibration curves are obtained by measuring the ratio of the peak heights of ethanol and the internal standard and plotting this ratio against the percentage of ethanol. This provides straight line plots. Hausdorff (6) found that peak heights are linear with concentration when symmetrical and sharp peaks are obtained, and that peak heights are generally as accurate as peak areas.

Acetone was selected as an internal standard after examining methanol, isopropanol, butanol, and pentanol. Accurate measurement of the sample is no longer critical with this method. Brealey, Elvidge, and Proctor (7) reported that doubling or tripling the sample size does not appreciably alter the ratio of the peak heights. Since a calibration curve is run with each unknown, it is not necessary to maintain precise operating conditions in the instrument from time to time. The slope of the curve will change slightly from day to day, but it is only necessary to run two concentrations of the standard solutions to determine the curve.

Ratios of ethanol peak heights to acetone peak heights for the synthetic mixtures of several preparations are shown in Table I. The resulting calibration curves are shown in Fig. 1.

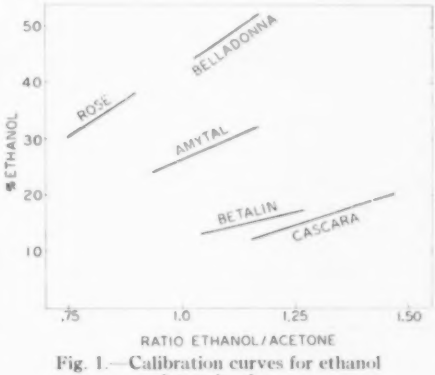

Fig. 1.—Calibration curves for ethanol determination.

PROCEDURE

Standard solutions containing acetone and a suitable amount of ethanol are prepared for each sample to be assayed. The sample is diluted with the same amount of acetone that is contained in the standards. Standards and samples are then chromatographed under the following conditions: instrument, Podbielniak Chromacon No. 9475-3V; column, 6 feet × 1/4 inch copper packed with 30% (w/w) polyethylene glycol 400 on 30–60 mesh Chromosorb and operated at 100°; sample inlet heater, temperature maintained at 300°; carrier gas, helium at an inlet pressure of 15 pounds/square inch and atmospheric pressure at outlet, flow rate 75 ml./min; pipet, Hamilton microliter syringe No. 705N, sample size 1–2 μL.; detector, Gow-Mac thermal conductivity cell, model 9285, detector current 68 milliamperes, output signal 32; recorder, Brown instruments division, Minneapolis-Honeywell Regulator Co. with a range of −0.2–2.0 mv. and a chart speed of 30 inches/hour.

The peak heights of the acetone and ethanol are measured accurately and the ratios calculated and plotted against the concentration of ethanol. From the standard plot the concentration of ethanol in the diluted sample is determined. This value, when corrected for the dilution with acetone gives the amount of ethanol present in the original sample. Table II shows the results obtained by the distillation and gas chromatography methods.

In addition to the above mentioned instrument, a Burrell Kromo-Tog, model K-2, with a filament-type detector and a shop-built instrument with a Victory Engineering Corp. thermistor-type detector, No. M 182, were also used. The results from these instruments compared very favorably with those shown in Table II.

SUMMARY

The gas chromatography method permits a sample containing ethanol to be assayed accu-

rately in less than one half hour as compared to at least one hour by the distillation method. No separations or special treatments are required in the former procedure; the sample needs only to be diluted with a known amount of acetone before being injected into the chromatograph. Results obtained by two analysts are very satisfactory.[1]

[1] The author wishes to thank Mr. G. W. Mills for assisting in the collection of some of these data.

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Metal Chelates and Diabetogenic Activity II*

Isoalloxazines

By WINTHROP E. LANGE, JAMES McMURTRY, and MERLE E. AMUNDSON†

In a further attempt to relate the chemical structure of various compounds to their diabetogenic activity, copper chelates of several isoalloxazines, and nitrogen-containing fragments (substituted anilines) of the isoalloxazine molecules, have been prepared. All of these compounds were found to be weak chelating agents. Only copper was found to be capable of forming stable 1:1 chelates. The chelates of dichloro-isoalloxazines as well as the chelates of the corresponding substituted aniline compounds were found to produce a hyperglycemic response in rabbits greater than that of alloxan or its metal chelates. The chelates of dimethyl-isoalloxazines and the corresponding substituted anilines as well as the unmetallized compounds were essentially inactive. These findings suggest that either chloro-substitution, or the presence of a sugar side chain is necessary for isoalloxazines to produce a hyperglycemic response as metal chelates.

KUHN AND WEYGAND (1) in 1934 were the first to experiment with isoalloxazine derivatives as antimetabolites. Since that time many isoalloxazines have been prepared and tested as: riboflavin antagonists (2), inhibitors of flavo-protein enzymes (3), and antifolinic compounds (4). Binet, Wellers, and Marquis (5) were the first to observe that large doses of various alloxazines produced a diabetogenic response in rats. This result is not surprising in view of the facts that the pyrimidine structure, considered essential for diabetogenic activity (6), is present and of the large doses employed.

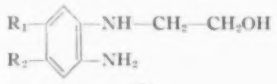
In the first paper in this series (7) we reported the preparation and blood sugar studies of various chelates of alloxan. At that time we also tested the chelates of two isoalloxazines, one of which produced a blood sugar response far greater than that of alloxan and the other showed only slight activity. In order to inquire further into the cause of the variations in response we obtained, copper chelates of various isoalloxazines and fragments of the isoalloxazine molecule have been prepared and tested in rabbits.

* Received August 21, 1959, from the Massachusetts College of Pharmacy, Boston, and the Division of Pharmacy, South Dakota State College, Brookings.
† Fellow of the American Foundation for Pharmaceutical Education, 1958–1959.
A portion of this work was abstracted from a thesis submitted by Merle E. Amundson as partial fulfillment of the requirements for the degree of Master of Science, 1959.
This study was supported in part by a research grant (A-1895) from the National Institutes of Health, U. S. Public Health Service.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

DISCUSSION

Methods of Synthesis.—In order to determine the influence of substitution on the benzene portion of the isoalloxazine molecule, two derivatives of aniline were prepared. The preparation of the

dichloro compound (Ia) has been reported by Barlow (3) as an intermediate in his synthesis of 6,7-dichloro-9-(2′-hydroxyethyl)-isoalloxazine. However, he did not isolate the compound. Evaporation of an alcoholic solution of the compound gave a product which could be purified and a correct analysis for carbon-hydrogen was obtained. A method

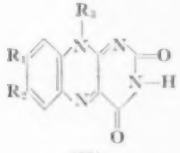


(I)

Ia $R_1 = Cl, R_2 = Cl$
Ib $R_1 = CH_3, R_2 = CH_3$

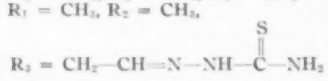
for the preparation of the dimethyl compound (Ib) involved the reaction of 4-chloro-5-nitro-*o*-xylene with 2-aminoethanol followed by hydrogenation.

The two corresponding isoalloxazines and a thiosemicarbazone derivative (IIa, IIb, and IIc) were prepared to determine the influence of the sugar



(II)

IIa $R_1 = Cl, R_2 = Cl, R_3 = CH_2—CH_2OH$
IIb $R_1 = CH_3, R_2 = CH_3, R_3 = CH_2—CH_2OH$
IIc $R_1 = CH_3, R_2 = CH_3,$



group found in the isoalloxazines previously described (7). Compound IIa had been prepared previously by Barlow (3) from Ia and alloxan. Compound IIb was prepared by cleavage of the sugar portion of riboflavin as reported by Fall and Petering (2). The thiosemicarbazone derivative (IIc) was also prepared by the method of Fall and Petering.

Chelate Formation.—Initially the preparation of the metal chelates was attempted in a manner similar to the preparation of metal chelates of riboflavin (8). However, the isoalloxazines required a pH of 12 for them to remain in solution in water. At this pH the major product obtained was the metal hydroxides. In order to prevent the formation of metal hydroxides, the solvent was changed to ethylene glycol and the pH adjusted to 9. Aqueous solutions of various metal salts were then added to the isoalloxazine solutions in equimolar quantities. A marked drop in pH which occurred in the absence of added alkali and immediate precipitation provides evidence for chelation rather than salt formation. Further evidence was provided by the absence of metal ions in the supernatant solutions as indicated by tests with ferricyanide. Unlike the riboflavin chelates (8) only chelates of copper could be obtained in a pure state. Thus, the sugar side chain of riboflavin must be involved in chelate formation of the hexacoordinate metals: namely, cobalt, nickel, and iron. This is not in agreement with the postulation of Albert (9) that riboflavin should helate like 8-hydroxyquinoline. If this were true the sugar group would not be involved in chelation with any metal.

The chelates of the 4,5-dichloro and 4,5-dimethyl-N-(2′-hydroxyethyl)-anilines were prepared in an ethanol-water mixture, due to the insolubility of the parent compounds in water, by the same procedure as described above. This procedure using cupric chloride gave immediate precipitation of a dark purple chelate with the dichloro compound and a pale green chelate with the dimethyl compound. As before only the copper chelate could be isolated in a pure state. Various other metals were attempted: namely, cobalt, nickel, iron (Fe^{++}), and zinc.

Structural Considerations.—The results of elemental analyses of the chelates, as shown in Table I, indicate a structure containing one metal atom per molecule of isoalloxazine or substituted aniline. The presence of water of chelation was shown by a loss of weight corresponding to approximately the calculated number of molecules of water without a change in color when the chelates were dehydrated at 250°. Only in the case of the copper chelate of IIb was any color change noted during the dehydration procedure. It darkened and appeared to decompose. In the case of the thiosemicarbazone derivative (IIc) the elemental analyses indicated a structure closely related to riboflavin (8) containing two metal atoms per molecule of isoalloxazine.

The ultraviolet absorption spectral characteristics of the isoalloxazines and their copper derivatives were compared in an attempt to show differences between the chelate and the parent compound. Little change in the position of the maximum absorption for the isoalloxazines occurred on formation of the chelates, as was previously observed for the chelates of riboflavin (10). The position of maximum absorption for the aniline compounds (Ia and Ib) shifted from 253 mμ to 240 mμ. No attempt at an explanation of this shift will be presented at this time.

Biological Results.—As shown in Table II, the administration of nondiabetogenic doses of the chelates produced a variety of responses in rabbits. The dichloro compounds, as chelates, produced increases in the blood sugar level far greater than did alloxan. The differences between the blood sugar increases caused by the chelates and the nonmetallized agents is striking. The dichloro compounds also were more toxic than alloxan. A dose greater than 50 mg./Kg. in most cases was fatal to the rabbits. The dimethyl compounds, both chelated and unchelated, in general were inactive. Riboflavin containing a sugar side chain produced a slightly greater blood sugar response as the chelate than alloxan. In the case where the sugar group of riboflavin was replaced by a thiosemicarbazone, or a hydroxyethyl group, neither the chelate nor the parent compound showed any appreciable effect.

These observations suggest that in some way, either chloro substitution or the presence of a sugar side chain on the isoalloxazine molecule are contributing groups in the production of blood sugar increases caused by the chelates of these compounds. Chelation alone is apparently not the final answer to the activity of various isoalloxazines, fragments of isoalloxazines, or of alloxan.

TABLE I.—ANALYSES OF THE METAL CHELATES[a]

Chelate	Formula		C, %	H, %	Cu, %	H_2O,[b] %	Yield	Color
6,7-Dimethyl-9-(2′-hydroxyethyl)-isoalloxazine, Cu	$C_{14}H_{12}N_4O_3Cu^+OH^-\cdot H_2O$	Calcd.	41.83	4.51	15.80	4.8		
		Found	42.64	5.67	12.94	...[c]	87	Brown
6,7-Dichloro-9-(2′-hydroxyethyl)-isoalloxazine, Cu	$C_{12}H_6Cl_2N_4O_3Cu^+OH^-\cdot H_2O$	Calcd.	33.93	2.38	14.96	4.3		
		Found	33.60	3.49	14.14	4.4	80	Green
Thiosemicarbazone of 6,7-dimethyl-9-formylmethyl-isoalloxazine, Cu_2	$C_{15}H_{11}N_7O_3SCu_2\cdot 4H_2O$	Calcd.	32.60	3.46	23.0	13.0		
		Found	32.26	5.07	21.8	12.2	50	Green
2-Amino-4,5-dimethyl-N-(2′-hydroxyethyl)-aniline, Cu	$C_{10}H_{15}N_2OCu^+Cl^-\cdot H_2O$	Calcd.	40.55	5.78	21.45	6.1		
		Found	40.00	4.43	22.80	5.8	34	Green
2-Amino-4,5-dichloro-N-(2′-hydroryethyl)-aniline, Cu	$C_9H_9Cl_2N_2OCu^+Cl^-\cdot H_2O$	Calcd.	28.79	3.19	18.86	5.4		
		Found	28.87	1.94	18.60	6.5	73	Purple

[a] The carbon-hydrogen analyses were determined by Weiler and Strauss, Oxford, England. The copper analyses were conducted by a standard ashing procedure. The chelates were dried *in vacuo* at 100° before analysis for carbon and hydrogen.
[b] This quantity is the loss of weight on dehydration at 250° for two hours.
[c] This compound gradually darkened during dehydration.

TABLE II.—BLOOD SUGAR DETERMINATIONS OF METAL CHELATES IN RABBITS[a]

Compound	Increase in Blood Sugar Level, mg % — Time, hr ½	1	2	3
Alloxan[b]	15	48	4	...
6,7-Dimethyl-9-(1-D-ribityl)-II,[c] Co[b]	23	72	50	−5
6,7-Dichloro-9-(1-D-sorbityl)-II, Co[b]	60	110	130	125
6,7-Dimethyl-9-(2′-hydroxyethyl)-II, Cu	0	−15	−10	...
6,7-Dichloro-9-(2′-hydroxyethyl)-II, Cu	57	85	80	37
6,7-Dimethyl-9-formylmethyl-II-thiosemicarbazone, Cu_2	0	0	−3	...
2-Amino-4,5-dimethyl-N-(2′-hydroxyethyl)-I, Cu	0	−7	−11	−13
2-Amino-4,5-dichloro-N-(2′-hydroxyethyl)-I, Cu	70	75	100	30

[a] The mode of administration in all cases was intraperitoneal, and the dosage 20 mg./Kg. In all cases the non-chelated compounds produced no change in the blood sugar level in rabbits.
[b] Inserted as comparison compounds (7).
[c] I = aniline, II = isoalloxazine.

EXPERIMENTAL

The melting points were taken on a Fisher-Johns block and are recorded as read. The elemental analyses were carried out at the Weiler and Strauss Microanalytical Laboratory, Oxford, England.

2 - Amino - 4,5 - dichloro - N - (2′ - hydroxyethyl) - aniline.—A solution of 5.0 Gm. (0.02 mole) of 2-nitro-4,5-dichloro-N-(2′-hydroxyethyl)-aniline (3) and 150 ml. of ethanol was obtained with the aid of heat. One-tenth gram of platinum oxide (Adam's catalyst, American Platinum Works) was added to the alcoholic solution and the solution was reduced in a Paar hydrogenator at 40 p. s. i. The reduction required about thirty minutes. The catalyst was removed by filtration and the alcohol was removed from the filtrate by vacuum distillation. Recrystallization of the product from water produced 3.5 Gm. (80% yield) of 2-amino-4,5-dichloro-N-(2′-hydroxyethyl)-aniline as purple needles, m. p. 128.5–130°.

Anal.—Calcd. for $C_9H_{10}Cl_2N_2O$: C, 43.43; H, 4.55. Found: C, 43.51; H, 4.68.

2 - Nitro - 4,5 - dimethyl - N - (2′-hydroxyethyl) - aniline.—A solution of 3.0 Gm. (0.01 mole) of 4-chloro-5-nitro-*o*-xylene (11) which contained an excess of 2-aminoethanol (5.0 Gm.) was refluxed in 100 ml. of pyridine for seventy-two hours. The pyridine was removed by heating under reduced pressure and the resulting solid was recrystallized from an ethanol:water:methanol mixture (4:4:2). A yield of 1.7 Gm. (52%) of 2-nitro-4,5-dimethyl-N-(2′-hydroxyethyl)-aniline was obtained as red needles which melted at 195–197°.

Anal.—Calcd. for $C_{10}H_{14}N_2O_3$: C, 57.14; H, 6.71. Found: C, 56.86; H, 6.66.

2 - Amino - 4,5 - dimethyl - N - (2′-hydroxyethyl) - aniline.—2 - Nitro - 4,5 - dimethyl - N - (2′-hydroxyethyl)-aniline was reduced in the manner described for 2-amino-4,5-dichloro-N-(2′-hydroxyethyl)-aniline. Recrystallization of the product from a 50% ethanol : water mixture gave a 66% yield of 2-amino-4,5-dimethyl-N-(2′-hydroxyethyl)-aniline as purple platelets; m. p. 160° (decompn.).

Anal.—Calcd. for $C_{10}H_{16}N_2O$: C, 66.64; H, 8.95. Found: C, 67.28; H, 9.06.

6,7 - Dimethyl - 9 - (2′ - hydroxyethyl) - isoalloxazine and Thiosemicarbazone.—These compounds were prepared from 6,7-dimethyl-9-formylmethylisoalloxazine hydrate according to the procedure reported by Fall and Petering (2).

6,7 - Dichloro - 9 - (2′-hydroxyethyl) - isoalloxazine.—This compound was prepared by modification of the methods of Barlow (3) and Holly, *et al.* (12). A hydrogenated solution of 12.8 Gm. (0.05 mole) of 2-nitro-4,5-dichloro-N-(2′-hydroxyethyl)-aniline (3) was filtered into a suspension of 14.8 Gm. (0.09 mole) of alloxan monohydrate and 33.6 Gm. (0.04 mole) of boric acid in 600 ml. of

acetic acid. The mixture was stirred intermittently at room temperature for three days and then filtered. The filtrate was evaporated to dryness *in vacuo*. The solid material obtained upon filtration and evaporation was suspended in boiling water to remove the boric acid. The solid remaining was collected and recrystallized from ethylene glycol. The green amorphous solid was washed with water and dried *in vacuo*. The yield was 11.9 Gm. (65%).

Preparation of the Metal Chelates.—The copper chelates of 6,7-dichloro, and 6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine as well as the thiosemicarbazone derivative were prepared by the same procedure.

To a mixture of the isoalloxazine (0.03 mole) in hot ethylene glycol was added sufficient 10% sodium hydroxide solution to dissolve the isoalloxazine. An aqueous solution of anhydrous cupric chloride (0.03 mole) was added slowly to the stirred solution along with sufficient 10% sodium hydroxide solution to maintain a pH of 9. After thirty minutes of further stirring, the solid which formed was collected and washed with water and acetone. Analyses, yields, and colors of the various chelates are shown in Table I.

The copper chelates of 2-amino-4,5-dichloro and 2-amino-4,5-dimethyl-N-(2′-hydroxyethyl)-aniline were prepared by dissolving 0.005 mole of each in 100 ml. of a 50% ethanol:water mixture. Sufficient 10% sodium hydroxide solution was added to bring the pH to 9. An aqueous solution of 0.85 Gm. (0.005 mole) of anhydrous cupric chloride was then added slowly over a period of ten minutes, along with sufficient 10% sodium hydroxide solution to maintain the pH at 9. After one hour of stirring, the product was isolated and purified by washing with water and acetone. Analyses, yields, and colors of the various chelates are shown in Table I. In no case did any of the chelates melt under 300°.

Determination of Blood Sugar Levels.—Normal blood sugar levels were determined in rabbits fasted for twelve hours prior to injection of the chelates or parent compounds. The sample for injection was passed through a No. 100 sieve and suspended in 3 ml. of water with the aid of a small quantity of sodium lauryl sulfate. The chelates were injected by the intraperitoneal route due to their poor solubility in water. After injection, blood samples were taken at one-half- to one-hour intervals, and the sugar content determined by the standard Micro-Folin-Wu method using a Bausch and Lomb "Spectronic 20" spectrophotometer.

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Determination of the Effect of the Hydrophil-Lipophil Balance on Drug Release from Bases by an *In Vitro* Colorimetric Method*

By ROGER Y. SPITTLE and CHARLES W. HARTMAN

Semisolid bases were prepared from liquid petrolatum and paraffin. Various emulsifying agents were added to produce a specific hydrophil-lipophil balance in the bases. A water-soluble dye was added to each base and its rate of diffusion into water was measured by a colorimetric method. The data obtained indicates that the HLB value of a base influences the rate of drug release from the base. The importance of surface area and drug concentration is demonstrated and other factors that probably influence drug release are suggested.

MANY INVESTIGATIONS have been conducted concerning drug release from ointment and suppository bases and the effect of the base on the therapeutic action of the ointment or suppository (1–9). The incorporation of emulsifying agents into ointment bases has been shown to have a definite influence on drug release (5, 7, 10, 11).

Hartman and LaRocca (6) found, using a colorimetric test for dye release from suppository bases, that emulsifying agents slightly increased the dye release. This was demonstrated by Whitworth (7) using a colorimetric method in

* Received August 21, 1959, from the University of Georgia, School of Pharmacy, Athens.
Presented to the Scientific Section, A. PH. A., Cincinnati Meeting, August 1959.

conjunction with an *in vivo* study of suppositories with rabbits.

Rhyne and co-workers (5), using a bacteriological assay method, found that various HLB in factors ointment bases gave different degrees of inhibition activity. This variation of inhibition with HLB was also found to be dependent on the physico-chemical properties of the antibiotic or antiseptic used.

A variation of HLB value within an ointment or suppository base should, upon administration, give a corresponding variation in the release of medicament from the base. At least part of the base should be emulsified with tissue fluids and cause a greater absorption of the drug.

The effects of nonionic emulsifying agents on the release of medication from hydrophillic ointment bases were tested by the agar plate method (12) and optimum release of medicament was obtained when the surfactant was employed in a 1 per cent concentration. These authors concluded that an increase in the surfactant concentration caused a decrease in the release of medication.

The purpose of this experiment was to determine, by an *in vitro* colorimetric method, the effect of the HLB on the release of a water-soluble dye from various bases. These bases were composed of mineral oil; paraffin; and 10 per cent, by weight, of several emulsifying agents having different HLB values. No attempt has been made to prepare ointment or suppository bases with physical properties or appearance acceptable for commercial use. However, efforts have been made to control those properties having an influence on drug release. The consistency of the bases was similar to the consistency of suppositories because it was hoped to compare work in the present studies with that from *in vivo* studies of suppositories using the same base.

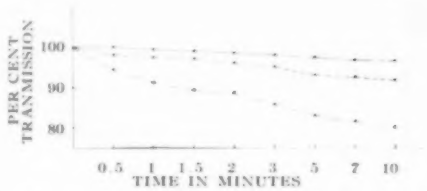

Fig. 1.—Per cent transmission of dye solution after release of dye from a base having an HLB value of 17.9, spread on different surface areas. Each base contained 10% of the emulsifying agent. ——— 0.4 cm. diameter, - - - - - - 0.6 cm. diameter, —·—·—·— 1.0 cm. diameter.

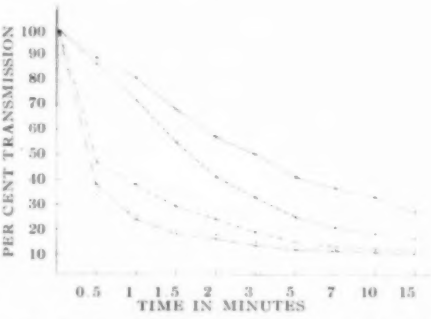

Fig. 2.—Per cent transmission of dye solutions after release of dye from a base with an HLB value of 10.5 containing different concentrations of dye. ——— 2% dye, - - - - - - 4% dye, ····· 6% dye, ·-·-·-·-· 8% dye, —·····— 10% dye.

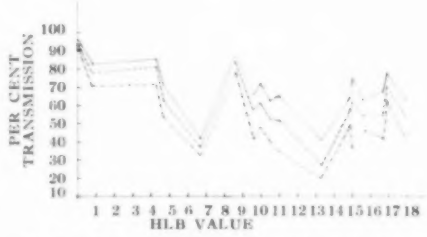

Fig. 3.—Per cent transmission of dye solutions after release of dye from bases having different HLB values. ——— Per cent transmission after three minutes, - - - - - per cent transmission after five minutes, —·—·— per cent transmission after ten minutes.

EXPERIMENTAL

Preparation of Bases.—The bases were composed of liquid petrolatum; paraffin; and 10%, by weight, of emulsifying agents. The emulsifying agents were the same as those used by Rhyne and Hartman (5) with the HLB values varying from 1.8 to 17.9. The consistency of the bases was adjusted by varying the per cent of paraffin and liquid petrolatum. All bases were prepared by fusion.

Two per cent amaranth water-soluble dye (FD&C Red No. 2) was added to the various bases by fusion. As shown in Fig. 2 this concentration showed a gradual release of the dye over a fifteen-minute period.

Colorimetric Method of Measuring Dye Release from Bases.—Fifteen milligrams of the base containing the dye was accurately weighed using an analytical balance and spread in a circle 1.0 cm. in diameter on 1.5 by 3.5 cm. strips of Whatman filter paper, No. 5. As shown in Fig. 1 the best ointment base diameter for the analytical procedure was 1.0 cm. By use of a No. 5 cork holding a small looped wire, the paper could be suspended in 18 cc. of water in a 19 by 105 mm. colorimeter tube. The paper did not contact the sides of the tube or the stopper, and was suspended slightly above the light path. The determination of the release of the dye from the bases was accomplished by measuring the per cent transmission of the solution on a Coleman junior spectrophotometer, model 6A. The per cent transmission readings on the colorimeter were made at definite time intervals after the base, on the paper

TABLE I.—PER CENT TRANSMISSION OF SOLUTIONS OF DYE RELEASED FROM BASES WITH AN HLB VALUE OF 17.9[a]

Emulsifying Agent, %	Time in Minutes								
	0.5	1	1.5	2	3	5	7	10	15
1	99.4	98.6	98.6	98.6	98.4	98.0	97.4	96.5	95.6
10	98.4	97.0	96.5	96.0	95.0	93.4	92.6	92.0	91.0
20	97.5	96.4	95.6	95.4	94.6	93.0	92.6	92.0	91.0
30	97.0	96.5	96.0	95.4	94.6	93.6	92.6	91.6	91.5
40	97.0	96.4	95.5	95.5	94.4	93.4	92.0	90.4	87.4
50	96.3	96.0	95.4	94.6	94.0	92.0	89.6	87.6	84.5

[a] Surface area 1.0 cm., 2% dye.

strip, came into contact with the distilled water in the colorimeter tubes. The time intervals at which readings were made are shown in Figs. 1, 2, and 3. The water had been placed in the colorimeter tubes prior to immersing the paper strips and was maintained at a temperature of 30° in a constant temperature bath until readings on the colorimeter were begun. A control base consisting of petrolatum only and 2% dye was treated in exactly the same manner. This control is recorded as an HLB base with a value of 0.

Figure 3 shows the per cent transmission of the dye solutions at three, five, and ten-minute intervals for the bases tested.

To determine the effect of a change in the concentration of the emulsifying agent bases were prepared containing 1, 10, 20, 30, 40, and 50% emulsifying agent with an HLB value of 17.9 and containing 2% dye. The results are given in Table I.

DISCUSSION OF RESULTS

Effect of HLB on Dye Release.—Results of the present work indicate a definite effect of the HLB factor on the release of medicament from bases. As shown in Fig. 3, with a variation of the HLB value, a change in the rate of release of the water-soluble dye was exhibited. The greatest rate of release of dye was observed at an HLB of 6.7 and 13.3. In general, the bases with lower HLB values gave a lesser rate of dye release than those with higher HLB values.

Petrolatum bases with no emulsifying agent gave very little dye release. This seems logical since their HLB values would be near zero.

The results found by the present colorimetric method using a water-soluble dye are in partial agreement with those found by Rhyne and Hartman (5). In their bacterial inhibition studies of ointment bases having different HLB values they found that neomycin sulfate, a water-soluble antibiotic, showed greater inhibition of bacteria in the bases with higher HLB values.

It is possible that several factors play an important part in the effect the HLB value has on drug release from a base. Three of these may be: the emulsification of the base with the body fluids, the distribution coefficient of the drug between the base and body fluids, and the intermolecular reaction between the drug and the surfactant.

Further studies using dyes less soluble in water should show, to some extent, the effect of drug solubility on release.

CONCLUSIONS

1. The HLB value of a base has a definite influence on the rate of release of medicament from the base.

2. The surface area of the base is a significant factor in the rate of drug release from bases.

3. The concentration of the drug in the base is of prime importance in the rate of release of the medicament from the base.

4. An increase in the concentration of the emulsifying agent in the base increases the rate of release of a water-soluble drug.

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Steroids I*

Synthesis of Steroid Nitrogen Mustards

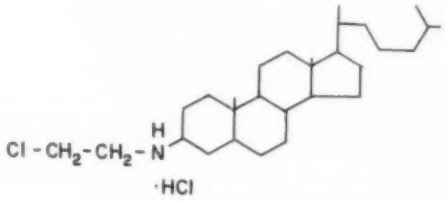
By ROBERT E. HAVRANEK† and NORMAN J. DOORENBOS

The synthesis of the steroid nitrogen mustards, 3α-(2-chloroethylamino)cholestane hydrochloride and 3β-(2-chloroethylamino)-cholestane hydrochloride, and their intermediates, 3α-(2-hydroxyethylamino)cholestane and 3β-(2-hydroxyethylamino)cholestane are described. These compounds were inactive in tumor tests administered by the National Serivce Center for Cancer Chemotherapy.

CANCER, which killed 255,000 Americans in 1958, is receiving more attention today than probably any other disease. Chemotherapy has been a useful tool for the treatment of patients with inoperable cancer. The most useful drugs have been metabolic antagonists, alkylating agents, and steroids.

In view of these facts, the synthesis of steroid alkylating agents for evaluation as cancer chemotherapeutic agents became of interest. A search of the literature revealed that some steroid nitrogen mustard alkylating agents had been synthesized. These compounds included N,N-bis(2 - chloroethyl) - 3 - amino - 5 - cholestene hydrochloride (1), N,N-bis(2-chloroethyl)-3-ergosterylamine hydrochloride (2), N,N-bis(2-chloroethyl)-3-stigmasterylamine hydrochloride (3), and 3β - [bis(2 - chloroethyl)aminoethyl)] - 5 - cholestene and its hydrochloride (4). Biological data were reported only on the last of these compounds. Toxicity studies of this compound were inconclusive because of its low solubility.

Formerly it was thought that a nitrogen mustard must have at least two reactive moieties per molecule for activity (5, 6). It was postulated that the nitrogen mustard must be capable of forming a cross link between two essential protein molecules, or between two portions of the same molecule. Recent facts, however, have demonstrated that monofunctional nitrogen mustards are useful in the treatment of cancer, although they are not nearly as active. Bond (7) has stated that the monofunctional nitrogen mustards should receive further study since they may have a better therapeutic index.



3β-(2-Chloroethylamino)cholestane Hydrochoride

The synthesis of nitrogen mustard derivatives of the 3-aminocholestanes with one reactive moiety per molecule was chosen for this investigation.

The 3-aminocholestanes were prepared from 3-cholestanone oxime using the stereospecific methods of reduction developed by Shoppee. Reduction with sodium and ethanol yielded 3β-aminocholestane (8, 9). Reduction by hydrogen in glacial acetic acid with a platinum catalyst yielded 3α-aminocholestane (10).

Pure samples of these amines were obtained only in small yield after long purification procedures including recrystallization and chromatography. Shoppee had similar difficulties (11). Probably these amines form intermolecular complexes with steroid impurities which are difficult to separate. Such complexes have been observed frequently with steroid alcohols. For these reasons, the impure amines were used in the next synthetic step. The products proved to be much easier to purify.

3β-Aminocholestane was condensed with 2-chloroethanol in the presence of sodium carbonate to yield 3β-(2-hydroxyethylamino)cholestane. 3-α-(2-Hydroxyethylamino)cholestane was prepared by condensation with the more reactive reagent, 2-bromoethanol. The nitrogen mustard derivatives were prepared by the reaction of thionyl chloride with these alcohols. Infrared spectra were used as an aid in the identification of these compounds.

3-Cholestanone oxime, the 3-aminocholestanes, and the four new steroids were submitted to the

* Received August 21, 1959, from the School of Pharmacy, University of Maryland, Baltimore 1.
This paper was awarded first prize for the Southern District in the 1959 Lunsford-Richardson Awards sponsored by Vick Chemical Co.
Abstracted from a thesis submitted by Robert E. Havranek to the Graduate Faculty of the University of Maryland, School of Pharmacy, in partial fulfillment of the requirements for the degree of Master of Science.
† Nonzema Foundation fellow (1958–1959).
Appreciation is expressed to Sterling-Winthrop Research Institute for the analytical data.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

National Service Center for Cancer Chemotherapy for tumor screening. None showed activity.

EXPERIMENTAL[1]

3β - (2 - Hydroxyethylamino)cholestane. — In a 2-L., three-necked flask, fitted with a stirrer, condenser, dropping funnel, and electric heating mantle, was placed 20 Gm. (0.051 mole) of 3β-aminocholestane dissolved in 400 cc. of purified benzene and 53 Gm. (0.5 mole) of anhydrous sodium carbonate.

The mixture was heated to reflux with rapid stirring and 40 Gm. (0.5 mole) of 2-chloroethanol added in four 10-Gm. portions during the first three hours of the reaction period. Refluxing was continued for ten hours more, the mixture cooled, and the sodium carbonate–sodium chloride precipitate filtered. Benzene was removed, under reduced pressure, with a Borg revolving evaporator. The warm residue was sprayed with ether and a white precipitate of 3β-(2-hydroxyethylamino)cholestane formed. The precipitate was filtered. Later 12 Gm. of unreacted 3β-aminocholestane was recovered from the ether solution.

The precipitate was dissolved in hot absolute ethanol and simultaneously treated with potassium carbonate and charcoal. The solution was filtered hot and the ethanol removed under reduced pressure. The residue was recrystallized from 4:1 acetone–ethanol. Cooling by refrigeration for a period of ten hours yielded 5.0 Gm. (54.9%) of white well-formed needles (yield was calculated on the basis of the starting material not recovered); m. p. 172.5–173.5°, $[\alpha]_D^{25}$ + 20° ± 0.1° (1% in chloroform).

Anal.—Calcd. for $C_{29}H_{53}NO$: C, 80.68; H, 12.38; N, 3.25. Found: C, 80.64; H, 12.24; N, 3.17.

3α - (2 - Hydroxyethylamino)cholestane. — 3α-(2-Hydroxyethylamino)cholestane was prepared from 20 Gm. (0.051 mole) of 3α-aminocholestane and 7 Gm. (0.056 mole) of 2-bromoethanol by a modification of the method used to prepare 3β-(2-hydroxyethylamino)cholestane. Absolute ethanol was used as the reaction solvent in place of benzene.

The purification procedure was the same as for the 3β epimer.

Recrystallization of the product from 4:1 acetone–ethanol yielded 9.0 Gm. (42.8%) of white platelets, m. p. 113.6–114.4°; $[\alpha]_D^{25}$ + 24.5° ± 0.1° (1% in chloroform).

[1] The melting points are corrected.

Anal.—Calcd. for $C_{29}H_{53}NO$: C, 80.68; H, 12.38; N, 3.25. Found: C, 80.83; H, 12.66; N, 3.17.

3β - (2 - Chloroethylamino)cholestane hydrochloride. — 3β - (2 - Hydroxyethylamino)cholestane (3.0 Gm., 0.0069 mole) and 100 cc. of freshly distilled benzene were placed in a 400-cc., two-necked flask fitted with a condenser and heating mantle. The mixture was heated to dissolve the reagent and 3.0 Gm. (0.02 mole) of thionyl chloride was added.

A precipitate immediately formed which was probably the benzene-insoluble amine hydrochloride salt. The mixture was refluxed one hour, cooled, and the excess thionyl chloride and benzene removed under reduced pressure in a Borg revolving vacuum evaporator.

The residue was dissolved in hot absolute ethanol and treated with 6 Gm. of Nuchar to remove the deep orange color. Subsequent filtration of the hot solution produced a clear, colorless solution that deposited colorless, fine needles on cooling with refrigeration. Successive concentrations of the mother liquor yielded more product to make a total yield of 1.7 Gm. (57%), m. p. above 300,° decompn.

Anal.—Calcd. for $C_{29}H_{53}Cl_2N$: C, 71.57; H, 10.98, total Cl, 14.57. Found: C, 71.48; H, 10.74; total Cl, 14.30.

3α - (2 - Chloroethylamino)cholestane hydrochloride. — 3α-(2-Chloroethylamino)cholestane hydrochloride was prepared from 2.0 Gm. (0.0046 mole) of 3α-(2-hydroxyethylamino)cholestane and 2.0 Gm. (0.13 mole) of thionyl chloride by the same procedure described for the synthesis of 3β-(2-chloroethylamino)cholestane hydrochloride. White needles were obtained in a total yield of 1.25 Gm. (63%), m. p. above 300°, decompn.

Anal.—Calcd. for $C_{29}H_{53}Cl_2N$: C, 71.57; H, 10.98; total Cl, 14.57. Found: C, 71.54; H, 11.26; total Cl, 14.40.

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Notes

A Note on the Inverse Isotope Dilution Analysis of Salicylic Acid*

By C. E. BRECKINRIDGE, Jr., and J. E. CHRISTIAN

Using the labeled derivative method of inverse isotope dilution, a procedure has been developed for the analysis of salicylic acid in submilligram quantities. A spectrophotometric method was utilized for the determination of the weight of the derivative. The sensitivity of three different methods of determining the radioactivity of I^{131} samples was investigated to determine the best counting procedure. Using equal aliquots of an I^{131} solution, a 2.5 mg./cm.^{2} window Geiger-Mueller counter showed a net activity of 1,385 counts per minute. A one inch NaI crystal scintillation counter registered a net of 31,806 while a liquid scintillation spectrometer[1] yielded a net rate of 88,550 counts per minute. Thus the liquid scintillation spectrometer was adopted for use in this work.

Radioactive iodine monocholoride (the reagent) was prepared as described by Swartz and Christian (1) with the exception that 20 mc. of I^{131} in the form of NaI^{131} in basic Na_2SO_3 was used, and only one distillation to complete dryness was carried out. These changes were made to increase the concentration and the specific activity of the product.

Samples of salicylic acid to be analyzed were prepared by dissolving a known weight of salicylic acid in glacial acetic acid. Appropriate aliquots of this solution containing the quantity of salicylic acid to be analyzed were placed in 7 cm. × 1 cm. test tubes and 1 ml. of the labeled reagent containing 425 mg. ICl was added. Since the quantities analyzed were 10 mcg. and 1 mcg., this represented a definite excess of ICl. The solution in the test tubes was heated in a water bath for twenty minutes at 70 to 80° with intermittent agitation by shaking. A known weight (2 mg.) of carrier (nonradioactive 3,5-diiodosalicylic acid) was then added. Heat was applied for five minutes to insure complete solution and intimate mixing. The solution was cooled to 50° and the derivative precipitated by adding distilled water dropwise. The mixture was digested at 40 to 50° for five minutes, then cooled to room temperature. The test tubes were placed in a centrifuge for five minutes, after which the supernatant liquid was discarded. The precipitate was washed with distilled water twice, dried, and crystallized from glacial acetic acid. The centrifuge was employed after each step. The crystals were washed with distilled water, dried, and dissolved in approximately 3 ml. of ethyl alcohol. Exactly 3 ml. of the resulting solution was pipetted into a quartz cuvet and the concentration of the solution was determined, using the Beckman DU spectrophotometer. A spectral-concentration curve for 3,5-diiodosalicylic acid was previously prepared at 328 mμ, the point of maximum absorbance. The samples were then transferred quantitatively to 20-ml. Wheaton vials using the phosphor solution for rinsing. Sufficient phosphor solution was added to give approximately 18 ml. and the activity was determined by liquid scintillation (Tri-Carb). The activity of the reagent was determined at this time using the same procedure. Toluene was used as the solvent, PPO (4 Gm. per liter) as the phosphor, and POPOP (0.1 Gm. per liter) as the wavelength shifter in the preparation of the phosphor solution. The results obtained at the 10 mcg. and 1 mcg. levels using this procedure appear in Table I. As little as 1-mcg. amounts of salicylic acid were determined with an average error of 15.7%.

Table I.—Results of Analyses of Salicylic Acid in 10 mcg. and 1 mcg. Amounts

Salicylic Acid Analyzed,[a, b] mcg.	Final Sp. A of 3,5-diiodosalicylic acid, c. p. m./mg.	Wt. of Salicylic acid by analysis,[c] mcg.	Error, %
10	6.777×10^3	9.1	9
10	6.533×10^3	8.8	12
10	7.921×10^3	10.7	7
10	6.605×10^3	8.9	11
10	6.533×10^3	8.8	12
10	6.457×10^3	8.7	13
1	6.088×10^2	0.81	19
1	6.752×10^2	0.90	10
1	6.592×10^2	0.88	12
1	6.672×10^2	0.89	11
1	5.769×10^2	0.77	23
1	6.088×10^2	0.81	19

[a, b] In each analysis, 2 mg. of carrier (3,5-diiodosalicylic acid) was added and the initial specific activity of the derivative was 5.323×10^5 counts per minute per mg.

[c] Calculated using the equation:

$$\text{wt.} = G_2 \frac{SpAf}{SpAi - SpAf} \times \frac{138.12}{389.94}$$

where: G_2 = weight of carrier added, $SpAf$ = specific activity of the carrier-diluted derivative, $SpAi$ = specific activity of the derivative as calculated from the specific activity of the reagent, 138.12 = the molecular weight of salicylic acid, 389.94 = the molecular weight of 3,5-diiodosalicylic acid.

* Received August 21, 1959, from the Bionucleonics Department, Purdue University, Lafayette, Ind.
Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.

[1] Packard Tri Carb liquid scintillation spectrometer.

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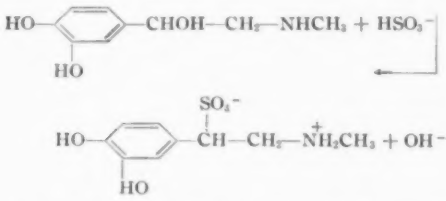
A Note on the Preparation of 1-(3,4-Dihydroxyphenyl)-2-methylaminoethane Sulfonic Acid from Epinephrine*

By LOUIS C. SCHROETER† and TAKERU HIGUCHI

KINETIC STUDIES (1, 2, 3) indicated that epinephrine reacts with sodium bisulfite or sulfite solution to yield a zwitter-ionic sulfonate:



The preparative value of this reaction and comprehensive physical constants of the product and its acetyl derivative have not been previously reported.

EXPERIMENTAL

Epinephrine hydrochloride, 0.025 mole (5.492 Gm.) and sodium bisulfite, 0.025 mole (2.602 Gm.) were dissolved in about 50 ml. distilled water; the pH was adjusted to 6.8 by the addition of standard sodium hydroxide. The solution was introduced into a three-necked flask fitted with a dropping funnel, reflux condenser, and a tube for flushing the solution with nitrogen. The reaction mixture (*ca.* 80 ml.) was refluxed for six hours while keeping it flushed with nitrogen. Standard hydrochloric acid (1 *N*) was added periodically to maintain pH range 6.7 to 6.9. After refluxing, excess water (*ca.* 65 ml.) was distilled from the solution. The white crystalline precipitate which separated on cooling the solution was recrystallized from 1 *N* hydrochloric acid and twice from distilled water. Yield: 5.28 Gm. (85.5%).

Anal.—Calcd. for $C_9H_{13}NO_5S$: C, 43.71; H, 5.29; H, 5.66; O, 32.36; S, 12.97. Found: C, 43.72; H, 5.79; N, 5.50; O, 31.90; S, 13.05. The product melted with decomposition at 263° (Kofler hot stage) and exhibited the following optical crystallographic properties: system, orthorhombic; class, dipyramidal; refractive indexes, $\alpha = 1.544$, $\beta = 1.660$, $\gamma = 1.696$; optic sign, negative; $2V = 55°$ (calculated from α, β, and γ). X-ray powder diffraction data shown in Table I were obtained using copper radiation with a nickel filter (1.5405 Ångströms). The pKa determined in water was 8.95. Ultraviolet spectrum in water shows maxima at 228 and 278 mμ; $\epsilon_{228} = 6{,}650$, $\epsilon_{278} = 2{,}850$.

1-(3,4-Diacetoxyphenyl)-2-methylaminoethane Sulfonic Acid.—Acetylation of 1-(3,4-dihydroxyphenyl)-2-methylaminoethane sulfonic acid was accomplished by suspending 2 m*M* (495 mg.) of the latter in 3.0 ml. glacial acetic acid. Two milliliters acetic anhydride and two drops concentrated hydrochloric acid were added to the solution and the latter refluxed for about twenty minutes. The white crystalline precipitate which separated on cooling the solution was recrystallized from glacial acetic acid. Yield: 413 mg. (62.5%).

TABLE I.—X-RAY POWDER DIFFRACTION DATA 1-(3,4-DIHYDROXYPHENYL)-2-METHYLAMINOETHANE SULFONIC ACID

d, A	I/I_1	d, A	I/I_1
8.34	0.43	3.64	0.32
7.40	0.48	3.52	0.26
6.18	0.90	3.40	1.00
5.58	0.85	3.33	0.34
5.29	0.87	3.21	0.46
4.69	0.35	3.09	0.29
4.58	0.33	2.81	0.58
4.32	0.60	2.65	0.39
4.17	0.41	2.44	0.34
4.01	0.82	2.24	0.31
3.75	0.83	..	..

TABLE II.—X-RAY POWDER DIFFRACTION DATA 1-(3,4-DIACETOXYPHENYL)-2-METHYLAMINOETHANE SULFONIC ACID

d, A	I/I_1	d, A	I/I_1
15.22	0.85	4.73	0.58
9.28	0.77	4.59	0.43
7.48	0.75	4.27	0.63
6.23	1.00	3.91	0.64
5.83	0.81	3.75	0.76
5.47	0.55	3.53	0.55
5.04	0.91	3.36	0.39
4.90	0.56	3.13	0.38
..	..	3.06	0.47

Anal.—Calcd. for $C_{13}H_{17}NO_7S$: C, 47.13; H, 5.13; N, 4.23; O, 33.80; S, 9.68. Found: C, 47.21; H, 5.16; N, 3.93; O, 33.67; S, 9.48. Acetyl determination: theory, 25.98%. Found, 26.74%. The derivative melted 212 to 213° (Kofler hot stage) and exhibited the following crystallographic properties: system, triclinic; refractive indexes, $\alpha = 1.480$, $\beta = 1.502$, $\gamma = 1.562$; optic sign, positive; $2V = 65°$ (calculated from α, β, and γ). X-ray powder diffraction data shown in Table II were obtained using copper radiation with a nickel filter (1.5405 Ångströms). The pKa determined in water was 8.30. Ultraviolet spectrum in water shows maxima at 263 and 269 mμ with a shoulder at 216 mμ: $\epsilon_{216} = 8{,}500$, $\epsilon_{270} = 411$.

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* Received December 7, 1959, from the School of Pharmacy, University of Wisconsin, Madison and the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.
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The authors wish to thank Dr. John W. Shell, The Upjohn Co., for helpful crystallographic suggestions and Dr. William A. Struck, The Upjohn Co., and members of his staff, for analyses.

A Note on the Biosynthesis of Clavine Alkaloids in *Claviceps purpurea* Strain 15B*

By L. R. BRADY and V. E. TYLER, Jr.

The data obtained with a replacement culture technique indicate two types of precursors are required for the formation of the ergoline nucleus in ergot strain 15B. L-Tryptophan was shown to be the most efficient aromatic precursor. DL-Phenylalanine and DL-β-phenyllactic acid were found to suppress alkaloid accumulation.

IT HAS BEEN ESTABLISHED that different clavine-producing strains of *Claviceps* exhibit different physiological responses (1). The two strains[1] which have been characterized in this laboratory show a marked contrast in their response to a reduced phosphate concentration in the growth medium and to additions of sucrose and DL-phenylalanine. The biosynthesis of the clavine alkaloids in strain 47A has been investigated using a replacement culture technique (2). It was shown that in this strain the ergoline nucleus is formed from two different types of precursors; an aromatic nucleus which may be derived from phenylalanine, tyrosine, or tryptophan, and a compound closely related to succinic acid or easily derived from it.

Since physiological studies had revealed that one of the amino acid precursors suppressed alkaloid accumulation in strain 15B, it was considered desirable to study the biosynthesis of the alkaloids in this organism by a replacement culture technique. This would separate a suppression resulting from gross physiological effects on growth from that associated more directly with the biosynthetic sequence which terminates with the clavine alkaloids.

EXPERIMENTAL

The ergot strain, strain 15B, which was employed in this investigation was isolated by Tyler from a sclerotium which had developed on *Agropyron semicostatum* Nees (3). The experimental conditions and manipulations have been described previously (2). The identity of the additions to the replacement media and the average alkaloid concentrations of the original growth media and the replacement media are recorded in Table I. Four replicate cultures were employed for each addition.

Paper chromatographic examination of the media indicated agroclavine was the only indole alkaloid which was present in detectable quantities. At the conclusion of the incubation period of the replacement media, tryptophan was detected in those cultures to which it had been added; a nonalkaloidal indole compound (R_f 0.85) was also observed in the media when this amino acid was present in combination with succinic acid. A detectable trace of phenylalanine remained in the media to which it had been added together with the organic acid. Tyrosine and phenylalanine were no longer present in other media to which they had been added.

DISCUSSION

The data are consistent with the conclusion that two types of precursors are involved in the biosynthesis of the clavine alkaloids (2). However, in strain 15B DL-phenylalanine is not a satisfactory aromatic precursor, and on an equimolar basis L-tryptophan is more efficient than L-tyrosine. The responses to the additions of the amino acids contrast with those elicited in strain 47A in which phenylalanine, tyrosine, and tryptophan were observed to be aromatic-type precursors of nearly equal efficiency.

The suppression of agroclavine accumulation by DL-phenylalanine and DL-β-phenyllactic acid is of considerable interest. Abe, *et al.* (4), reported that phenylacetic acid caused a similar retardation in the accumulation of clavine alkaloids in replacement culture; the ergot strain employed by them had also been isolated from an *Agropyron* host. These observations suggest that phenylalanine and certain structurally related compounds act as clavine alkaloid antimetabolites in strain 15B.

TABLE I.—CONCENTRATION OF CLAVINE ALKALOIDS IN CULTURE MEDIA

Replacement Medium Mineral Solution plus	Growth Medium, γ/ml., Agroclavine	Replacement Medium, γ/ml., Agroclavine	Clavine Alkaloids, %, Estimated Factor	Corrected Content of Replacement Medium, γ/ml., Agroclavine
DL-Phenylalanine	96.3	36.6	100	36.6
No additions	83.0	40.1	100	40.1
DL-Phenylalanine, succinic acid (1)	114.6	40.6	100	40.6
DI-Phenylalanine, succinic acid (2)	155.5	50.8	100	50.8
L-Tyrosine	87.1	56.4	100	56.4
DL-β-Phenyllactic acid, succinic acid	126.0	61.8	100	61.8
Succinic acid	154.7	92.3	100	92.3
L-Tyrosine, succinic acid	166.6	106.0	100	106.0
L-Tryptophan	94.0	257.7	50	128.9
L-Tryptophan, succinic acid	159.7	274.2	50	137.1

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* Received November 16, 1959, from the Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle 5.
Abstracted from a dissertation submitted to the Graduate School of the University of Washington by L. R. Brady in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
[1] Cultures of these two strains of *Claviceps* have been deposited with the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

A Note on the Effect of Gibberellic Acid on *Azotobacter indicus**

By LEO GREENBERG and JOHN TIRPAK

ALTHOUGH gibberellins are widely known as growth stimulants in higher plants, no effect on microorganisms was demonstrated in studies by Brian, *et al.* (1), and Ciferri and Bertossi (2). Interest in the influence of gibberellins was aroused by the report of Lu, *et al.* (3), that numerous colonies of *Azotobacter* appeared on sodium albuminate agar plates upon cultivation of soil treated with ethanolic gibberellic acid. In view of the fact that, to the best of our knowledge, no laboratory study of *Azotobacter* and gibberellins has appeared in the literature, such an investigation was undertaken.

Purified gibberellic acid was obtained commercially (Sigma Chemical Co.) and *Azotobacter* species purchased from the American Type Culture Collection. Preliminary studies showed that only *Azotobacter indicus* (ATCC 9540) grew in a diffuse fashion throughout the test medium with a minimum of pellicle formation or discoloration by pigment formation or waste products.

Dilutions of gibberellic acid were made with sterile isotonic saline which was shown to have no detectable effect on the normal growth of the organism. *A. indicus* was maintained on nitrogen-free mannitol agar in Kolle flasks and a standard liquid inoculum prepared by adding one loopful of surface growth to 25 ml. of sterile mannitol-tap water medium assigned a value of 100% transmittance at 420 mμ on a Coleman spectrophotometer. This standard inoculum was incubated at 25° until an absorbance of 80% transmittance was achieved, a process usually requiring about seventy-two hours.

Two sets of optically matched tubes containing 25 ml. of sterile mannitol-tap water medium and assigned the arbitrary value of 100% transmittance at 420 mμ of the spectrophotometer were used. Five milliliters of the appropriate dilution of gibberellic acid was added to one set of tubes and 5 ml. of sterile saline was added to the control set. All tubes then received 0.1 ml. of bacterial inoculum and kept well mixed at 25°. Changes in optical transmittance were recorded daily for ten days.

Repeated trials showed that in almost all cases, a distinct increase in growth as measured by decrease in optical transmittance could be demonstrated in the presence of gibberellic acid. A typical comparison of experimental to control series can be seen in Fig. 1. To determine if this stimulatory effect could be demonstrated in other media, a quintuplicate run using Ashby's medium and gibberellic acid in a concentration of 1000 mcg./ml. was performed. After ten days' incubation with the test organism, experimental tubes gave a mean transmittance value of 72.7 ± 0.8% compared to a control value of 77.3 ± 1.7%. This difference is significant, P being less than 0.05.

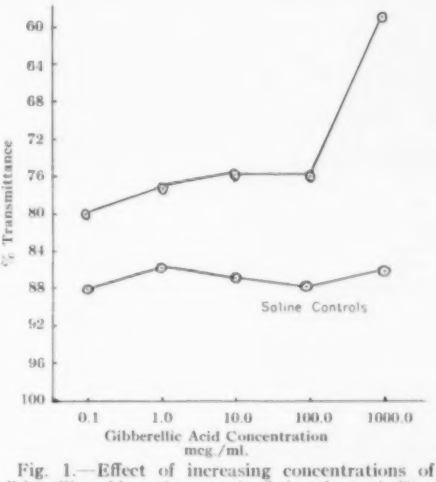

Fig. 1.—Effect of increasing concentrations of gibberellic acid on the growth of *Azotobacter indicus* compared to replicate saline controls (ten days incubation).

On the basis of our determinations, it seems likely that solutions of gibberellic acid have a stimulating effect upon *Azotobacter indicus*, but the lack of linearity and the occasional variations in extent of stimulation indicate that this effect is a complex physiological one.

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* Received January 19, 1960, from the Brooklyn College of Pharmacy, Long Island University, Brooklyn, N. Y.

Book Notices

Chemical Analysis. Vol. 10. The Chemical Analysis of Air Pollutants. By MORRIS B. JACOBS. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. xviii + 430 pp. 15 × 23 cm. Price $13.50.

This volume of a series of monographs on analytical chemistry and its applications presents detailed methods for the determination of the kind and amount of air contaminants. The presentation for each method follows the pattern: 1. General methods for sampling with procedures for the determination of air and gas volume, quantity, and velocity. 2. Analysis of the settled particulate matter and also the suspended particulate matter. 3. Methods for the analysis of gaseous and vapor contaminants of the atmosphere, including radiochemical methods. Appended are tables of analytical data relating to suspended particulate matter in the air, sootfall, gaseous contaminants, and motor vehicle exhaust gas composition. A subject index is included.

Soviet Research in Pharmaceutical Chemistry. Part I, Pharmaceutical Chemistry; Part II, Medicinal Chemistry; Part III, Pharmacognosy. English translation. Consultants Bureau, Inc., 227 West 17th St., New York 11, N. Y., 1959. 603 pp. 21 × 27 cm. Paperbound. Price Part I, $12; Part II, $45; Part III, $10; Complete collection $60.

These additions to the Chemistry Collection Series include translations of selected reports that have appeared in Soviet publications. The pharmaceutical chemistry part includes nine papers on ion exchange and its applications, particularly relating to the behavior of ion exchange resins with antibiotics. Fourteen additional miscellaneous reports are included. Part II includes thirteen papers on anti-infective compounds, eight papers on steroids and related compounds, thirty-five papers on heterocyclic compounds, and twenty miscellaneous reports. Part III, entitled Pharmacognosy, includes seventeen papers on plant alkaloids, one on pectic substances of sunflower, and one on corelborin P.

Fermente-Hormone-Vitamine. Band I. Fermente. 3rd ed. By ROBERT AMMON and WILHELM DIRSCHERL. Georg Thieme Verlag, Stuttgart, Germay, 1959. Agents in U. S. and Canada, Intercontinental Medical Book Co., 381 Fourth Ave, New York 16, N. Y. xx + 564 pp. Price $22.85, Subscription price $19.45

This third edition, in three volumes, is the first revision since 1943. Volume 1, on ferments or enzymes, is, of necessity, a completely rewritten text. It attempts to relate significant relationships between biological catalysis and enzymes. This is developed with regard to individual and group enzyme activities. The book is intended for use by biochemists, medicinal chemists, and others in related chemical fields.

Lymphocytes and Mast Cells. By MARGARET A. KELSALL and EDWARD D. CRABB. The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1959. xvi + 399 pp. 15 × 23 cm. Price $8.

This book is a comprehensive treatise on two cells: Lymphocyte—a specialized cell which synthesizes, stores, and transports nucleoprotein for maintenance, growth, or secretion by other cells; and Mast cell—chief significance is ability to synthesize, store, and release histamine and heparin, but its production of mucopolysaccharide and its possible relation with serotonin is also discussed. A bibliography of 1,142 references cited in the text and a subject index are appended.

Organic Reactions. Vol. 10. Edited by ROGER ADAMS. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1959. vii + 563 pp. 15 × 23 cm. Price $12.

This volume of "Organic Reactions," the tenth since the series was started in 1942, continues the comprehensive treatment of selected subjects. The ninth volume of the series was reviewed in THIS JOURNAL, **47**, 155(1958). The present volume covers: The coupling of diazonium salts with aliphatic carbon atoms; the Japp-Klingemann reaction; and a very complete treatment of The Michael reaction. This latter chapter covers 379 pages and includes 1,045 references. The excellent style of presentation developed for this series is continued. A subject index for Volume 10 and cumulative author and chapter heading indexes are appended.

The Preparation of Medical Literature. By LOUISE MONTGOMERY CROSS. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1959. xx + 451 pp. 15 × 22.5 cm. Price $10.

This book is designed to be used as a desk reference for details on practical techniques in the preparation of medical reports, articles, and books; in which physicians and allied scientists communicate clinical experience and scientific investigation to their colleagues. Writers for publication in any scientific field will find this book useful. Chemists should note that abbreviations for names of journals are from the "Quarterly Cumulative Index Medicus" and the "Current List of Medical Literature." As of January 1960, these two reference publications were replaced by "Index Medicus" (new series) published monthly the the National Library of Medicine, and annual cumulations of the index will be published by AMA. The journal abbreviations in the new "Index Medicus" follow, with some variations, those used in "Quarterly Cumulative Index Medicus." Writers of material for publication in the chemical fields should rely on the abbreviations recommended by *Chemical Abstracts*.

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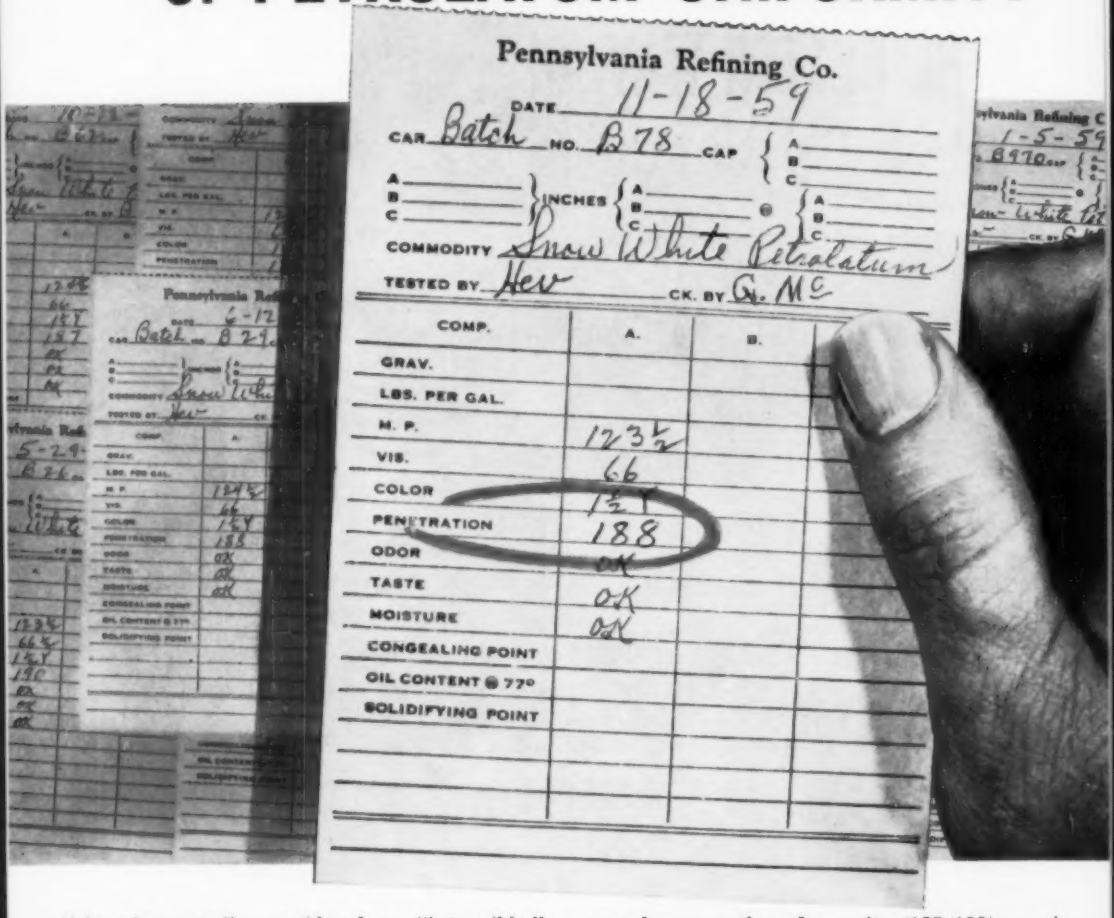

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